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A Physical Approach to the Toxicity of Fine Particles

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Abstract

Nanotechnology is a growing field that represents a large financial market with many industrial and consumer applications. Nanoparticles are widely known to be toxic when inhaled, regardless of chemical composition. This is in contradiction with currently held theory which ascribes a chemical cause to the toxicity of fine particles. This study proposes that fine and ultra-fine particle toxicity is caused by the presence of trapped charges in defects at the particle surface. Through a physical mechanism of action, these charges are transferred generating reactive oxygen species and inducing biological damage. Samples of metal-free phthalocyanine were heated in I_2, O_2, or EtOh vapor in order to withdraw or inject electrons at point defects respectively to alter their electronic properties. *In vitro* toxicity of both fumigated and untreated materials was then analyzed using THP-1 cell assays in collaboration with the University of Montana. Results were then compared to determine the contribution of trapped charges at defects to toxicity. Treated H_2Pc was found to cause a higher degree of oxidative stress than untreated material in all cases. Toxicity was also shown to increase with grinding and decreased particle size. The purpose of this research is to create a framework for developing a physical set of criteria useful in determining the toxicity of nanomaterials that may be analyzed without the use of expensive, time-consuming biological methods.

1. Introduction

Nanotechnology, the study and application of nanoparticles, is utilized in consumer products ranging from icing in donuts, sunscreen, toothpaste, and cosmetics[1]. The industry has come to represent a $2.4 trillion portion of the global marketplace.[2] Nanoparticles (NP), those with at least one dimension between 1-100 nm, are particularly notable in the fact that they possess entirely different physiochemical properties than macro particles of the same material. NP have a very large surface area per unit mass. This causes them to be much more reactive than molecules of identical chemical composition on the macro scale. This increase in surface area also has the effect of increasing the amount of defects in NP structures, such as an irregularity in the otherwise consistent crystal structure, vacancies between atoms, and the inclusion of foreign molecules such as metal catalysts often used to synthesize NP. While features such as increased reactivity are useful properties when developing nanotechnologies, this reactivity also manifests itself biologically as toxicity.[1]

Toxic effects have been well documented, and include the ability to damage DNA leading to cancer and oxidative damage to the lungs through the formation of Reactive Oxygen Species (ROS).[3] It has been shown that NP are able to cause chronic pulmonary inflammation, pulmonary fibrosis, and lung tumors in rats.[4,5] As a result of inflammatory conditions, the removal of these particles by alveolar macrophages (AM) is impaired, leading to a buildup of particles in cells, connective tissues, and lymph nodes. This "particle overload" also causes the release of inflammatory signaling molecules from phagocytes, the production of fibroblasts leading to fibrosis, and epithelial damage leading to cancer. Studies have shown that NP are more likely than larger particles to generate free radicals and cause oxidative stress to the body due to their increased surface area per unit mass. The generation of the reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), oxygen (O_2•), and hydroxide (OH•) radicals, is considered a primary

contributor to nanotoxicity, though the exact mechanism of ROS generation *in vivo* remains unknown. When AM fail to remove NP from the lungs, transcription factors within the cell are activated and ROS are produced along with a host of other signaling factors that ultimately culminate in apoptosis.[5]

When considering NP toxicity, current theory holds that the mechanism of action is a function of the particle's chemical composition, however, there are drastic chemical differences between NP such as TiO_2 and carbon black, though they have been shown to exhibit similar levels of toxicity.[5] This would seem preclude a chemical mechanism of action as an explanation for this shared characteristic. Additionally, NP of identical chemical composition and size distribution are able to exhibit varied toxic effects dependent on surface features.[5] This would indicate that physical properties of fine particles may play a primary role in their toxicity.

Studies evaluating the toxicity of NP have yielded conflicting results. Some studies have concluded that NP induce an inflammatory, fibrotic reaction in lung tissue with genotoxic effects on epithelial cells in vivo, while others observed little or no toxic effects. These contradictory conclusions may be attributed to the wide variety of synthesis methods available which produce NP of varying sizes and defect concentrations. Additionally, NP are often altered post-synthesis to vary purity, adsorptive nature, aspect ratio, surface reactivity, hydrophilic properties, and surface functionalization. This wide variety of NP properties has made the development of universal criteria governing their toxicity difficult.[6]

A study performed by Muller et al.[7] analyzed the role of defects in lung toxicity of multi-walled CNTs by introducing defects through grinding. The ground material was then analyzed concurrently with other samples heated to 2400 ˚C to reduce the amount of defects and metal impurities by shrinking them in a process known as annealing. The relative amount of defects contained within samples was characterized using Raman spectroscopy and adsorption microcalorimetry, and samples were then administered to rats through inhalation. Results showed that the ground, unheated samples were far more toxic than those heated to 2400 ˚C demonstrating that a decrease in defect concentration through annealing is able to decrease toxicity. This data shows a strong correlation between defect concentration and toxicity.[7]

Studies analyzing silica (SiO_2) NP have shown that toxicity is strongly dependent on surface defects. Further, adverse reactions *in vivo* are able to be prevented using anti-silicotic drugs, polyvinylpyridine-N-oxide and aluminum lactate, which inhibit active sites on the surface of the structure formed by defects in the NP crystal structure.[8] These studies indicate that physical properties of fine particles strongly contribute to their toxicity, and that the increase in toxicity correlated with a decrease in particle size is due to the increase in defect concentration at the particle surface.

The role of defects in the generation of ROS is easily explained by a well-known phenomenon. Semiconducting NP demonstrate a trapping phenomenon whereby electrons are localized to specific electron wells known as non-equilibrium free charge carriers (NFCs) which are able to participate in electrical transport. An injection current may be generated by the injection of carriers into the system of a NP.[9] Neutral point defects and impurities are able to capture NFCs, localizing them and charging the capture center. Once a carrier has been localized, it may either recombine with an opposite sign carrier or be released. This occurs in the conduction band for electrons and the valence band for holes.[9]

The wave functions of all states in a perfect crystal extend over the whole crystal and have the same probability amplitude in every cell. When an impurity of other defect is introduced, periodicity is broken, and localized states are allowed, usually appearing in the fundamental gap. Impurities in semiconductors have varying effects, depending on the energy levels and are able to contribute to the forbidden band. Shallow level impurities create donors levels that are almost entirely ionized to the conduction band at room temperature. Deep level impurities however contribute negligibly to current carrier concentrations. In most cases, their function is to facilitate the recombination of electrons and holes by providing a level somewhere in the middle of the band gap. For an electron in the conduction bands to drop into an empty state in the valence bands, an amount of energy equal to the band gap must be released. Deep impurities generally create acceptor levels that facilitate recombination. The electron or hole is able to recombine by dropping through multiple excited states.[10] These levels are illustrated below (Figure 1).

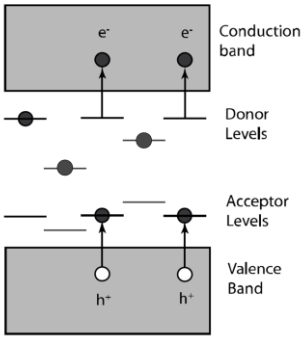

Figure 1. Illustration of donor and acceptor levels created by impurities in a crystal structure.[11]

NFCs are strongly influenced by the presence of localized states created by impurities, point defects, surface states, dangling bonds, and localized stresses which are able to act as trapping centers. Atoms with the same valence as a substituent within a crystal structure can easily be substituted as an impurity, but an atom with a smaller radius than the rest of the crystal structure will bind electrons more tightly forming an electron trap. Vacancies in a lattice will enable the valences of surrounding atoms to trap electrons. Gasses are able to be adsorbed onto particle surfaces, bonded through Van Der Waals forced, forming a surface state that may either trap electrons or holes. The majority of traps located on a particle surface are composed of adsorbed atoms, dangling bonds, or misfit-induced internal stresses. In many nanoscale species, the number of atoms on the surface of the particle is the same order of magnitude as the total number of atoms, all of which are able to act as traps or recombination centers. [10]

This trapping phenomenon is shared by a variety of semiconducting nanomaterials including carbon nanotubes (CNTs) and metal-oxides such as TiO_2, SnO_2, ZnO and SiO_2 and has primarily been studied in terms of the application of NP for use in electronic devices.[9] Contributions of trapping and detrapping could be key to understanding why a variety of nanomaterials exhibit similar toxic effects despite widely varying chemical composition. By gathering data on electric properties of a substance in conjunction with defect concentration and toxicity, the determination of this contribution becomes possible. The purpose of this project is to demonstrate the influence of the electronic behavior due to NFCs formed at defects on the toxicity of NP.

It has been demonstrated that electronic traps can be either populated or emptied through absorption of gas phase molecules. This would theoretically alter the toxic effects demonstrated by NPs by altering the relative distribution of NFCs. An unequal distribution of NFCs raises the threshold voltage required for recombination to occur, increasing the lifetime of localized charges.[10] Creating a larger distribution of either positive or negative charges, makes recombination less likely and increases the overall amount of defects which are able to contribute to the generation of ROS. Injecting or withdrawing electrons into electron wells has been shown to alter the charge distribution of surface defects. Physisorbed O_2 molecules on solid surfaces are known to act as electron traps. This is also known to inhibit isotopic exchange when triggered by detrapped electrons. This phenomena has been observed in several types of semiconducting materials and is utilized for various types of electronics.[10]

Phthalocyanine was chosen as the main focus of this study due to its semi-conductive properties and ubiquitous use in industry. They are either metal-free (H_2Pc) or contain a coordinated metal center (MPc) and are commonly used in dyes, solar cells, vapor sensors, and as catalysts.[12] Pc most commonly acts as a p-type semiconductor, and displays little conductivity unless doped by gas phase oxidants like O_2.[13]

Sensor studies using H_2Pc thin films have suggested physisorption of O_2 molecules takes place before chemisorption. Physisorption occurs below 40 kJ/mol dominated by weak van der walls forces attracting the molecules to the surface of Pc through an induced dipole. Once close enough, chemisorption begins to dominate as a result hydrogen bonding between O_2 and the interior N-H of the Pc molecule, shown below (Figure 2). O_2 was found to bind competitively with all analytes studied including CO, NO_2, and NH_3 significantly affecting the response and recovery of the film.[14]

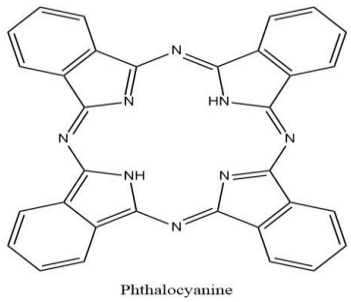

Figure 2. Molecular structure of H_2Pc.

Studying the effects of adsorbed O_2 on electronic properties, Yasunaga et al. performed an experiment in which O_2 was diffused into PbPc using a thermal treatment for 10 hours at 230ºC. The thermoelectric power of the material measured afterwards indicated that positively charged carriers were generated by the adsorbed O_2 by accepting an electron from a neighboring PbPc molecule and releasing a hole-like carrier, increasing the density of positively charge defects.[15]

In a similar study of isotopic exchange in solid cobalt complexes doped with radio-Co^{2+}, Kaillat & Nath proposed that the excitation energy required to induce isotopic exchange and thermal annealing in the solid state is derived from the recombination of holes and electrons from multiple levels. CoPc was heated in the presence of EtOH or O_2 at various time intervals followed dissolution of the samples in H_2SO_4. The rate it which each reformed was measured by radiochemical analysis of each material's activity as it reformed using a scintillation counter. O_2 was seen to inhibit the isotopic exchange due to the depopulation of electron donors on the particle surface, resulting in decreased conductivity. In contrast, EtOH was shown to increase the rate of isotopic exchange by populating electron wells, increasing overall conductivity.[16]

Proceeding along these lines, this study thermally treated H_2Pc in O_2, EtOH, and I_2 in order to alter the distribution NFCs on the surface. Adsorbed O_2 and I_2 reduce conductivity of the material by withdrawing electrons at defects. Theoretically, by removing localized electrons responsible for the generation of ROS, the overall concentration of localized positive charge will increase. In contrast, treatment with EtOH, shown to increase conductivity, injects electrons at defects leading to a higher concentration of localized negative charge. Once treated, the toxicity of these materials was evaluated to determine the effect localized surfaces charges at defects have on fine particle toxicity.

Once established, a connection between conductivity and toxicity could allow for materials to be tested for toxicity using physical methods. There is an urgent need for a set of broadly applicable criteria to assess the risks of nanomaterials. The formulation of a testing strategy that is able to be used on a wide variety of materials has proven difficult.[17] The results of this research could enable the population of NFCs to be used as a metric for high throughput methods of testing toxicity that can be performed cheaply and quickly without biological assays.

2. Methods

2.1 Materials Characterization

β-29H,31H-Phthalocyanine (98%) was acquired from Sigma Aldrich and an α/β mixture of H_2Pc (98.4%) from Tokyo Chemical Industry America. IR spectra of materials were obtained using a Thermoscientific Nicolette iS10 spectrometer confirming both molecular identity and structural conformation of the H_2Pc crystals obtained.

Size measurements of each material were obtained using a Nikon Eclipse LV 100 POL optical microscope and NIS-elements software. β-Pc samples were observed to be needle-shaped with average dimensions of 4 µm width by 46 µm in length. The α/β Pc mixture was formed of rounded grains averaging 50 µm wide by 81 µm in length. Images of both samples are shown below (Figure 3).

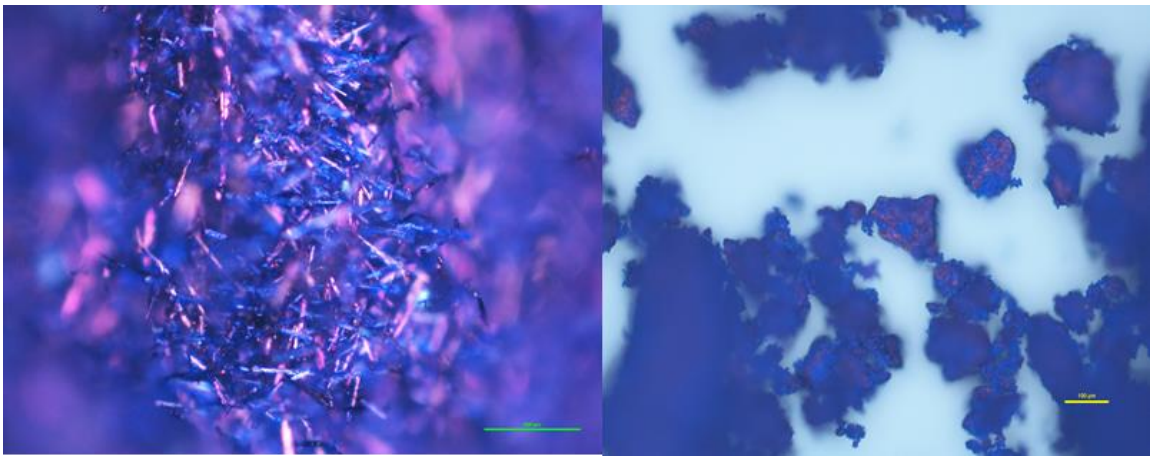

Figure 3. Left, 98% β-H_2Pc magnified 10x using optical microscopy, right, 98% α/β-H_2Pc magnified 20x using optical microscopy

2.2 Thermal Treatment with O_2 and EtOH

In an attempt to alter the distribution of NFCs, 50 mg samples were treated in either ambient O_2 or EtOh using a VWR 1410 vacuum oven at 150 ºC for 24 hours. Ambient treatment with O_2 was performed by placing samples in the oven with an open vent, allowing the circulation of O_2 at atmospheric concentration. In order to treat samples with EtOh, 30 mL of 99% EtOH was placed in the oven with the sample and allowed to evaporate. To prevent adsorption of O_2, a vacuum pump was used to reduce the pressure within the oven to 6 cm.hg and returned to atmospheric pressure with N_2 gas 3 times, reducing O_2 concentration to < 0.1%. Once treated, all samples were refrigerated at 10ºC.

2.3 Iodine Treatment of Samples

Iodine treatments were administered by placing open vials of I_2 (99%) and H_2Pc within sealed containers. One treatment was placed within the vacuum oven and heated at 60 ºC for 24 hours, and a second was left at room temperature for 20 days.

Both iodinated samples were then placed into a glass bomb, and a flow of N_2 gas was passed through the enclosure to allow excess iodine to sublimate without exposing the samples to oxygen.

2.4 Grinding of Samples

To reduce the size of Pc particles, samples were ground using an Across International QM 3-P04 interplanetary ball mill with zirconia milling chamber and milling balls (Φ6 50, Φ10 8). Samples were placed into a stainless steel milling chamber and sealed. A vacuum pump was then used to remove atmospheric O_2 from the chambers by reducing pressure to 6 cm.Hg and refilling with N_2 gas 3 times. Samples were then milled at 12 minute intervals for 4 hours. Optical microscopy was performed according to previous method. Milled particles of β-Pc (batch 2) were observed to have much larger size distribution than unmilled particles, ranging from .70-17 µm with average dimensions of 5.2 x 3.8 µm (Figure 4). The size distribution of α/β Pc (batch 3) changed similarly to .49-25 µm with average dimensions of 5.0 x 3.2 µm.

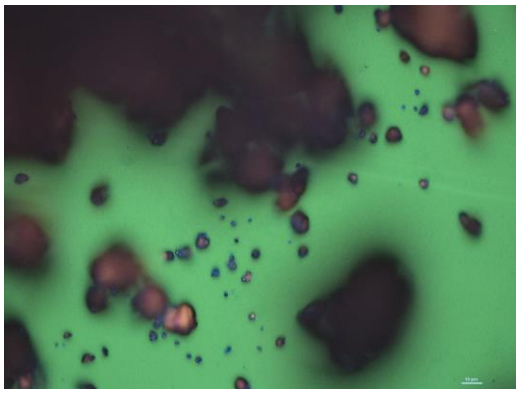

Figure 4. Optical Microscopy image of H2Pc after being milled for 4 hours

Sample batch 4 was prepared by milling α/β Pc according to the previous method for 6 hours. This reduced the size distribution to .35-22 µm with average dimensions of 1.5 x 1.2 µm.

Milled samples were handled and stored in an inert N_2 atmosphere contained within a polyethylene glove bag or a glove ball in order to prevent atmospheric O_2 from adsorbing to the surface of the material.

2.5 Cytotoxicity Measurements

Once treated, samples were sent to Dr. Andrij Holian at the University of Montana for toxicological testing. Dose-response lactate dehydrogenase (LDH), mitochondrial activity assays (MTS), and interlekin-1 β (IL-1 β) (Cell Titer 96 and Cell Tox 96 from Promega) were then performed in triplicate on each batch of three samples using THP-1 cells, a human monocytic cell line derived from leukemia cells. Assessments were performed after a 24 hours period of NP incubation within the cells. Uptake of NP was quantified using TEM and CytoViva. Studies were performed at 25 µg/ml measured at 1, 4, and 24 hours.

Increases in LDH and decreases MTS during the assays are associated with cell death caused by the material. An increase in IL-1 β indicates and signals the activation of an inflammatory pathway initiated by oxidative stress caused by ROS.

3. Results/Discussion

Initial LDH assays performed on β-H_2Pc in batch 1 displayed below (Figure 5) showed little difference between the treated and untreated materials, likely because the particles were too large to be taken up into the cell.

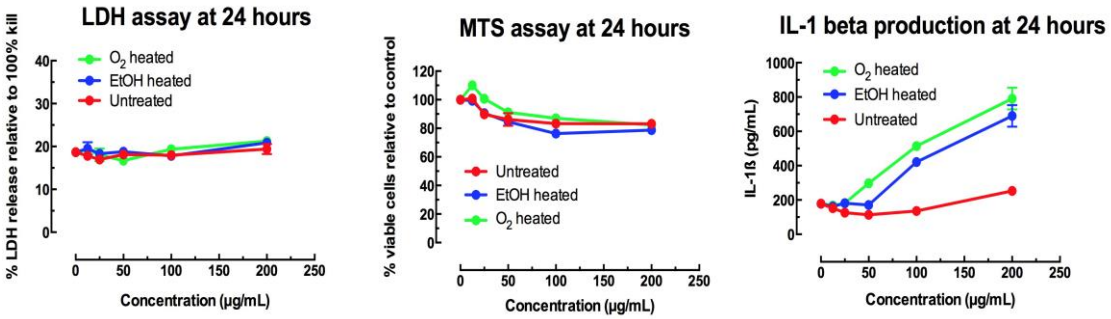

Figure 5. Batch 1 a) LDH Assay at 24 hours b) MTS Assay at 24 hours c) IL-1 β production at 24 hours.

Similar concentrations of LDH and MTS in the case of both treated and untreated samples indicate little difference in the amount of cell death. The IL-1 β assay indicates the activation of the NLRP3 inflammasome complex activated by phagolysosomal membrane damage which has been theorized to be the result of the generation of ROS and RNS. This assay clearly demonstrates that the untreated H_2Pc caused less oxidative stress in the THP-1 cells. Additionally, EtOH treatment was found to be only slightly less damaging than O_2 treatment with both demonstrating similar trends over time.

Batches 2-3 were composed of ground β and α/β H_2Pc particles respectively. Similar trends in toxicity were observed for both batches in MTS and IL-1 β assays. These results are located below (Figure 6). Both treated particles were shown to exhibit a higher degree of oxidative stress compared to untreated particles. Additionally, the concentration of IL-1 β overall was generally higher than in unground particles. This result was predicted by the proposed model of this study, which holds that the toxicity of a materials increases with an increase in surface area and concentration of surface defects in the material.

Batch 3, composed of a mixture of α/β H_2Pc, was shown to be slightly more toxic with a greater decrease in MTS activity and a higher concentration of IL-1 β indicating a higher degree of oxidative stress within the cells. This is likely due to the decreased size distribution of the particles, a small fraction of which may have been small enough to pass through the cell membrane. Additionally, ethanol treated particles in this batch seemed to be more toxic than similarly treated H_2Pc in the β conformation, though this deviation is well within the margin of error, denoted the error bars in Figure 6.

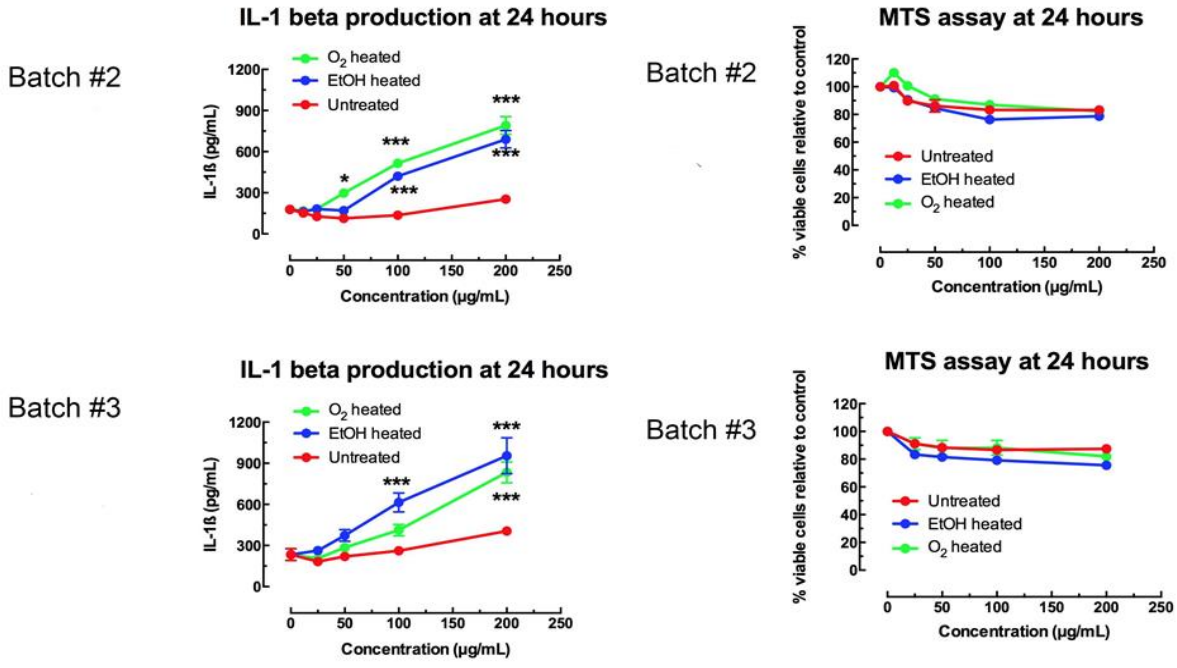

Figure 6. Batches 2 and 3 IL-1 β and MTS assay results

Results for batches 1-3 support the hypothesis that it is possible alter a material's cytoxicity by changing the distribution of NFCs in fine particles, but fail to validate the proposed model. As H_2Pc is classified as a semiconductor, it was hypothesized that adsorbed O_2 at defects on the particle surface may decrease toxicity of the particles by withdrawing electrons and depopulating electron wells. Conversely, it was postulated that EtOH, acting as an electron donor would inject electrons into the system, increasing the rate and which NFCs recombine, and enhancing toxicity as a result. The results obtained in this study indicate that both treated materials exhibit similar degrees of oxidative stress. It is possible that adsorbed O_2 is able to act as a reagent, contributing to the generation of ROS and increasing oxidative damage to phagolysosome cell membranes despite the altered population of NFCs.

To determine whether the observed oxidative stress observed in O_2 treated materials resulted from bulk O_2 adsorbed at the surface, sample batch 4 included iodine treated samples, one heated and the other treated at room temperature. These results are illustrated below (Figure 7).

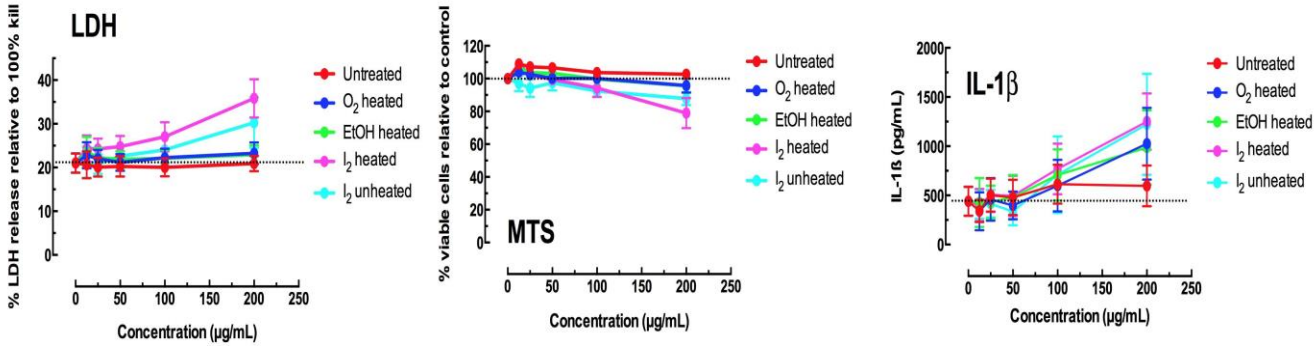

Figure 7. Batch 4 a) LDH Assay at 24 hours b) MTS Assay at 24 hours c) IL-1 β production at 24 hours.

Both I_2 treated samples exhibited much higher degrees of both cell death and oxidative activity. At this particle size, a larger degree of oxidative stress was observed for all particles, including the untreated samples. The observed amount of cell death remained similar in untreated, O_2 and EtOH treated materials, while I_2 treated materials were observed to be considerably more cytotoxic overall. Additionally, increased oxidative stress was observed for the untreated material. This supports the notion that an increase in surface defects achieved through a decrease in particle size is one of the primary contributing factors to fine and ultra-fine particle toxicity.

Results of the I_2 treated materials indicate that localized positive charges are responsible for the generation of ROS in the O_2 treated material as well. Despite the fact that H_2Pc is widely regarded as a p-type semiconductor, it is likely that the effect of the adsorbed molecules altered the charge distribution so sufficiently to prevent localized charges from recombining, resulting in a higher concentration of positively charged defects able to participate in the generation of ROS.

4. Conclusion

This study has successfully demonstrated that the toxicity of a molecule can be modulated by altering the distribution of NFCs at surface defects in fine particles. It has also demonstrated further that toxicity increases with a decrease in particle size as shown in previous studies. These results support the hypothesis that the toxicity of fine particles is influenced the physical properties of a material.

The model initially proposed by this study failed to accurately predict the increase in toxicity seen for I_2 and O_2 treated H_2Pc. More research is required to determine if the interaction of holes injected into the material with the biological environment is responsible for the observed increase in toxicity. Preliminary results obtained from I_2 treated materials seem to indicate that the injection of holes into H_2Pc increases sample toxicity greatly. This would support the notion that holes are able to interact similar to trapped electrons in the generation of ROS species within cells.

To determine conclusively whether observed toxicity is due to the electronic character of defects or simply a result of bulk molecules adsorbed at the particle surface, further studies will be pursued. Using space-charge-limited-current, it should be possible to inject electrons or holes directly into the material without the introduction of impurities, making it possible to clarify the roles of each.

Other variables may have contributed to the failure of the proposed model to accurately predict toxicity. While Pcs are generally known to be p-type semiconductors, the conductive properties of the materials used were not analyzed. In future, use of a semiconducting material deliberately synthesized or characterized as either p-type or n-type may demonstrate the proposed model more clearly.

Attempts to modulate toxicity in particles through thermal treatment to date have not yet been shown to diminish toxicity. Despite this, we have found that toxicity is indeed able to be correlated to the physical and electronic properties of a molecule, laying the ground work for the establishment of a more comprehensive model to be developed in future.

5. References

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