Final Progress Report

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Project Title: Role of Surface Chemistry in the Toxicological Properties of Manufactured Nanoparticles

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Abstract

Globally, both industrial workers and the general public are being exposed to a variety of damaging respirable environmental contaminants on a daily basis. Poor air quality has been linked to chronic respiratory diseases and instances of these diseases are increasing worldwide. The last decade has seen the birth of nanotechnology in which the novel properties of engineered nanoparticles are being exploited for advancements in technology and medicine. The driving force for making nanoparticles is to obtain materials with novel electrical, mechanical, optical properties. Knowledge of the reactivity of the surface sites will provide new strategies for reducing risks of occupational hazards. The focus of this project was to study the correlation between the surface reactivity of nanoparticles and their toxicity. The experimental approach focused on several classes of extensively used nanoparticles, with biological endpoints involving oxidative stress and inflammatory responses of aluminosilicate catalysts, titania, quantum dots, silver and carbon. Titania and aluminoslilcates were found to be non-inflammatory. For carbon, the inflammatory response seems to track the size of the particle. Macrophages exposed to synthesized particulates impregnated with iron resulted in secretion of the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) along with an increase in endothelial cell adhesion molecule expression. In addition, surface iron speciation and content were a driving factor of oxidative stress induced macrophage activation. Quantum dots (QDs) are semiconductor nanocrystals that have found use in bioimaging, cell tracking and drug delivery among other applications. Research regarding the toxicity and fate of QDs in biological systems is relevant to better understand cell/nanoparticle interactions. We have developed an inexpensive, time efficient microwave based synthetic protocol to develop high quality QDs. The biological characterization of these QDs was used to better understand the endocytosis of inorganic nanoparticles by macrophages and associated cellular responses. The QDs associate with scavenger receptors and also are internalized via clathrin dependent endocytosis. Using real-time, live cell imaging, we found that QDs interact with the cell surface within minutes and progress through the endocytic pathway to the lysosomes upon internalization. As demonstrated by assays of LDH release, dead red staining and annexin V staining, we found these QDs to be minimally toxic to exposed macrophages. In addition, QDs induced small but significant levels of TNF-α secretion. Using dye-entrapped aluminosilciate zeolites, we have developed an intracellular oxygen sensor. Human monocyte-derived macrophages internalized the submicron-sized $Ru(bpy)_3^{2+}$-zeolite crystals, and intracellular oxygen concentrations initiated by zymosan-mediated oxidative burst could be monitored by measuring the emission from $Ru(bpy)_3^{2+}$ by confocal fluorescence microscopy. The antimicrobial properties of patterned aluminosilicate zeolite films containing nanosilver were successfully demonstrated using *E-coli* bacteria as the model system and complete bacteria eradication was noted within 120 minutes.

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Section 1

Highlights/Significant Findings

- For carbon particles, surface iron speciation and content were a driving factor of oxidative stress induced macrophage activation.
- QDs were found to be minimally toxic to exposed macrophages. In addition, QDs induced small but significant levels of TNF-α secretion.
- Titania and aluminoslilcates particles were found to be non-inflammatory.
- Intracellular oxygen concentrations initiated by zymosan-mediated oxidative burst in macropahges could be monitored by measuring the emission from dye-aluminosilicates by confocal fluorescence microscopy.
- Antimicrobial properties of patterned aluminosilicate zeolite films containing nanosilver were successfully demonstrated.

Translation of Findings

- Inhalation of carbon nanoparticles with soluble and/or redox active iron species should be avoided.
- Cadmium-based quantum dots exhibit small, but significant inflammatory properties.

Outcomes/Relevance/Impact

Speciation of iron present on environmentally relevant particles and the bioavailability are the determining factors in their toxicity, and elemental analysis or even the valence state is not a sufficient predictor of toxicity.

Patterned zeolite films containing nanosilver are an effective antimicrobial agent and can be incorporated into a variety of systems, including fabrics, biomaterials, filters and thus can serve a wide range of uses.

Low cost, rapid synthesis and biological characterization of quantum dots.

Section 2 :Scientific Report

Background

Human beings have been exposed to nanoparticles from anthropogenic sources, especially derived from industrial and automotive processes. Considerable toxicological and epidemiological literature is available that documents health effects of particles.
The last decade has seen the birth of nanotechnology in which the novel properties of engineered nanoparticles are being exploited for advancements in technology and medicine. The driving force for making nanoparticles is to obtain materials with novel electrical, mechanical, optical properties.

Nanosized particles (NSP) are defined as including the class of particles < 100 nm, nanoparticles (NP) to be engineered particles (< 100 nm) of spherical size, and nanofibers, nanorings etc. to indicate other shapes. Ultrafine particles (UFP) are NSP's that are not engineered, e.g., products of industrial combustion process. As we progress from bulk to NP's of the same material, the significant differences are increase in particle numbers per unit mass, increase in surface area and alteration of surface chemistry since the number of unsaturated atoms (with broken bonds) increase exponentially with decreasing size.

Considerable research is being carried out on creation of very diverse NP's, including metals, ceramics, semiconductors, carbon, oxides etc, the surface structure varying with not only specific types of materials, but also within a particular class of materials, as different morphologies and crystal structures are possible. Thus, any generalization about safety and health issues of NP's without attention to the specific material will not be possible.

The influence of surface chemistry on biological activity is evident from in-vivo rodent studies with TiO_2 (20 nm) and polytetrafluoroethylene (PTFE ~18 nm).[8,9] Amongst manufactured nanoparticles, CNT have recently attracted attention because of their fibrous nature and persistence.[10-12] In- vitro studies with single-walled (SWCNT) and multi-walled (MWCNT) carbon nanotubes have shown evidence of oxidative stress, including free radical formation, oxidative products and reduction in antioxidants. The contribution of transition metal impurities that are usually present in carbon nanotubes towards the biological effects has not been examined. Several studies have suggested oxidative stress as a mechanism by which inhaled particulates mediate biologic responses, and oxidative stress has been associated with inflammatory responses.[6,13]

Objectives of the research program

Focus of nanoparticle toxicity is often on the increased surface area of the smaller size particles.[1] But, the role of local surface chemistry of nanoparticles in causing its toxicity is equally important.[2] As particles get smaller, the major structural change that occurs is the number of unsaturated bonds on the surface. The presence of these "broken" bonds will alter reactivity of the particles with other species including interactions with other particles, provide new sites for

coordination of metal ions, undergo redox chemistry, acid-base reactions and possess altered dissolution properties.

This research program is built around the hypothesis of surface reactivity:
Hypothesis 1. The quantifiable differences in surface reactivity of nanoparticles as compared to micron-sized particles of similar composition cannot solely be explained by increase in surface area of the nanoparticles.
Hypothesis 2. Enhanced oxidative stress and inflammatory response mediated by nanoparticles over micron-sized particles is related to the altered surface reactivity of nanoparticles.

In particular, we focus on surface reactivity as manifested in acidity, redox chemistry, complexation/coordination of transition metal ions and metal ion-mediated reactive oxygen species generation (Fenton reaction). We examined three manufactured nanoparticles: aluminosilicate catalysts (zeolites), titania and carbon, the latter also including single walled carbon nanotubes (CNT), as well as quantum dots and silver nanoparticles. Exposure to these nanoparticles can occur during manufacturing and use. Zeolites are crystalline aluminosilicates used extensively as catalysts and catalyst supports in the chemical and petrochemical industries with commercial impact extending to billions of dollars. There is increasing interest in nanocrystalline zeolites because of enhanced transport through the smaller particles, though not yet commercially used. Titania finds use in many applications, including catalysts supports, in paints and consumer products. Its structural, catalytic, electrical and optical properties are the driving force for applications. Carbon particles find major use in rubber reinforcement, whereas CNT are finding applications based on their mechanical and electrical properties.

The experimental program in general consisted of

1. Obtaining and synthesis of nanoparticles of zeolite Y, TiO_2 (rutile), carbon and CNT, QDs and Ag.
2. Characterization of bulk structure (spectroscopy, diffraction), morphology (electron microscopy) and surface area.
3. Surface centered chemical reactions including acidity, redox properties, metal binding and Fenton reactivity.
4. Analysis of oxidative stress upon internalization by macrophages or pulmonary epithelial cells by gene array techniques and assay of oxidation end products.
5. Analysis of inflammatory responses by measuring cytokines, NF-κB activation, and endothelial adhesion molecules.

Research Findings

1. Toxicological Properties of Manufactured Titania and Zeolite Particles

We did not find a noticeable increase in inflammatory behavior with titania and zeolite particles and they were not pursued further.

2. Toxicological Properties of Carbon Nanoparticles

Carbon black nanomaterials are consumer products with worldwide production of millions of tons per year. Printex™ 90 and Flammruss™ 101, both produced by the Evonik Degussa Corporation, are representative of a furnace black and a lamp black, respectively. In the furnace

black process, carbon particles are produced by spraying a pre-heated stream of petroleum feedstock into a closed furnace, where it is partially combusted in a controlled atmosphere. Lamp blacks are produced by burning a pool of oil feedstock in ambient conditions, and the soot is cooled and collected. Printex™ 90 is representative of a carbon black that is used in pigments, and Flammruss™ 101 is representative of a carbon black that is used as a filler to increase the durability of rubber. As nanoparticles with primary particle diameters of approximately 14 nm and 100 nm, both Printex™ and Flammruss™ carbon blacks are inhalable. Exposure to nanoparticles may be a risk to the health of their manufacturers and consumers. The surface functionality found on carbon particles includes acidic and basic functional groups, carbon and oxygen centered free radicals, and traces of redox-active metals.

Inflammatory responses following exposure of synthesized carbon nanoparticles to human macrophage and endothelial cells were employed as indicators of particulate biological activity. Hundred nanometer carbon particles (nC) with and without nonextractable surface-bound iron were synthesized using a templating approach, and human monocyte-derived macrophages (MDM) were exposed to various concentrations of these particulates. Supernatants recovered from MDM 24 h postexposure were assayed for the inflammatory cytokine tumor necrosis factor- (TNF) by a quantitative ELISA and tested for their ability to induce expression of intercellular adhesion molecule-1 (ICAM-1) on human endothelial cells (EC) by immunofluorescence flow cytometry. Data generated by these experiments demonstrated that nC-Fe was far more biologically active than nC. In addition, the chemical reactivity of nC-Fe toward decomposition of hydrogen peroxide to form hydroxyl radicals was significantly higher than that of nC and correlated well with the increase in the strength of the inflammatory response, though a direct proof of creation of hydroxyl radicals in the biological system is not provided. Comparison with micrometersized carbon and carbon–iron particles suggests that the chemical and biological reactivity is correlated with surface area. However, with the manufactured C particles (~20 nm), the C and C-Fe samples both show minimal biological response. Anomalous chemical and biological reactivity of nanoparticles of similar bulk composition prepared by different methods is a new finding and must be related to different surface functional groups. In-vitro oxidative stress and inflammatory responses upon phagocytosis by macrophages and pulmonary epithelial cells correlated with surface structure/binding/redox properties of nanoparticles.

The Fenton reaction is a means of generating very reactive ·OH from H_2O_2. The method of detection ·OH was spin trapping with DMPO, followed by EPR spectroscopy. The characteristic 4-line EPR spectrum is indicative of the formation of ·OH. For multiwalled carbon nanotubes (MWCNT), the complexed iron is not Fenton active, but with ascorbic acid, there is leaching of the iron resulting in Fenton activity. The chemical and biological properties of iron-loaded manufactured carbon nanoparticles (Flammruss 101) were contrasted with those of an iron-loaded synthetic carbon particle. X-ray photoelectron spectroscopy was used to characterize the iron on the carbon particles. Production of hydroxyl free radicals *via* the Fenton reaction was monitored by electron paramagnetic resonance spectroscopy. The iron-loaded synthetic carbon particles produced a positive Fenton response, whereas, the iron-loaded manufactured carbon particles did not. The source of the Fenton activity of the synthetic carbon particles is proposed to be a soluble iron compound that was formed during the synthesis of the particle. A likely candidate for the soluble iron species is Fe_2F_5, which was synthesized and its properties

examined. Higher toxicity of Fe_2F_5 towards murine macrophages compared with other simple iron salts was attributed to soluble iron that was stabilized by the fluoride ligand. The cytotoxicity of manufactured carbon particles towards murine macrophages decreased or remained unaltered upon impregnation with iron compounds.

To further simulate ongoing exposure to air pollution, we plated these same nano-sized carbons on 24-well plates then exposed the entire plate to ozone for 4 hours before immediately introducing murine alveolar macrophages (MHS) in serum-free media for 24 hours to these particulates. The supernantants were harvested and clarified by centrifugation before being assayed by ELISA for TNF-α and for cytotoxicity by colorimetric quantitation of LDH release. We found that ozonation reduces the cytotoxicity of the particulates. Further, TNF-α secretion from the macrophages is significantly reduced, as compared to non-ozone exposed carbon. Interestingly, this TNF-α inhibition is lost when ozonated nanoparticles are exposed to ambient for 24 hours. The ozonated particles were examined by infrared spectroscopy to investigate the changes in surface functionality. Transmission infrared spectroscopy was performed on a film of carbon black particles deposited to the calcium fluoride window of an FTIR gas cell from suspension in acetone. Our results show the best resolution to date of the evolution of surface oxygen functionality that occur when carbon black is exposed to ozone.

3. Toxicological Properties of Manufactured Quantum Dots

Quantum dots (QDs) are highly fluorescent, semi-conductor nanocrystals. Since their discovery in the early 1980's, the production and applications of QDs has quickly increased. In September 2008, market-research company BBC research of Wellesley Massachusetts predicted that the market for products relying on quantum dots would grow from $28.6 million in 2008 to $721 million by 2013. With such dramatic growth, availability of consumer products containing quantum dots is inevitable. Quantum dots have already made their mark in the biomedical industry as they are commonly employed in intra cellular and extra cellular bioimaging. Quantum dots are being used to fine-tune the colors of light-emitting diodes and as a backlight for flatscreen televisions. With such utility in technological applications, it is likely that QDs will be ubiquitous. Additionally, as the production of QDs increases, the environmental fate and transport of quantum dots becomes an important issue. Research regarding the toxicity and fate of QDs in biological systems is relevant to better understand cell/nanoparticle interactions

Although QDs outperform traditional organic fluorophores, the conventional synthesis is time consuming and costly. We have developed an efficient, inexpensive one-pot synthesis protocol for effective, biologically functional QDs. Our QDs contain a CdSe core surrounded by dual shells, the innermost containing CdS and the outer shell containing ZnS. The synthesis of our QDs incorporated a solution of $Zn(NH_3)_4^{2+}$ as a zinc source, which was used to optimize the initial solution of $Cd_4Se_1MPA_{20}$. The ensuing solutions were then microwave irradiated, and the end result was QDs that were crystalline in structure, approximately 7nm in size and emitted at a median wavelength of 550nm.

We next sought to characterize these QDs in a biological system. Cells from a murine alveolar macrophage (MHS) cell line were incubated with increasing concentrations for QDs and were fixed using 4% paraformaldehyde for 60 minutes and counterstained with DAPI before being

visualized using confocal microscopy. When compared to commercially available QDs (Invitrogen QD585), our QDs had a more punctate appearance intracellularly. We were also able to establish that the QDs are internalized quickly (< 20 minutes) and are excluded from the nuclei. In addition, we found these QDs to be safe, as exhibited by their lack of cytotoxicity in cell membrane integrity assays assaying LDH release.

Because of their apparent cellular internalization and intracellular localization, we decided to further biologically characterize these synthetic QDs. Specifically, we are interested in their mode and kinetics of internalization and intracellular compartmentalization. We have used murine macrophages to characterize the cytotoxicity and inflammatory effects of microwave synthesized QDs. . Internalization of our QDs can be partially blocked by a non-specific scavenger receptor ligand, indicating that internalization of our QDs is at least somewhat receptor-mediated. Murine alveolar macrophages were incubated for one hour with 100ug/mL polyinosinic acid, a non-specific scavenger receptor ligand. Then, increasing concentrations of QDs suspended in culture medium containing poly(I) or medium alone were added to the wells. The macrophages were allowed to incubate for 24 hours before being washed 3 times with PBS. The cells were then gently scraped, stained with Sytox Red to determine live vs. dead cells and assayed for fluorescence using flow cytometry. The QDs associate with scavenger receptors and also are internalized via clathrin dependent endocytosis. Using real-time, live cell imaging, we found that QDs interact with the cell surface within minutes and progress through the endocytic pathway to the lysosomes upon internalization. As demonstrated by assays of LDH release, dead red staining and annexin V staining, we found these QDs to be minimally toxic to exposed macrophages. In addition, QDs induced small but significant levels of TNF-α secretion. The biological characterization of these QDs can be used to better understand the endocytosis of inorganic nanoparticles and associated cellular responses.

4. Nanosilver Zeolite Films on Micropatterned Porous Alumina and Its Application as an Antimicrobial Substrate

In this study, we focused on synthesis of patterned zeolite films and the potential application of a silver-derived form of this film as a biocidal agent. The synthetic strategy has been to develop a patterned porous alumina substrate using soft lithographic methods. These patterns have dimensions in the range of 5-100 microns. Previously patterned PDMS and PMMA molds were used to define surface microfeatures on the alumina supports. Zeolite films (2-3 microns) were then grown on the alumina using a seeding process followed by secondary growth. Electron microscopy showed that the zeolite film followed the pattern of the alumina substrate. Silver nanoparticles were grown on the surface of the zeolite film by reduction of the Ag^{+} - exchanged zeolite with aqueous hydrazine. The antimicrobial properties of the patterned zeolite films were successfully demonstrated using *E-coli* bacteria as the model system; complete bacteria eradication was noted within 120 minutes. Such patterned zeolite films could potentially be incorporated into a variety of systems, including fabrics, biomaterials, filters and thus can serve a wide range of uses.

5. Entrapment of Ionic Tris(2,2′-Bipyridyl) Ruthenium(II) in Hydrophobic Siliceous Zeolite: O_2 Sensing in Biological Environments

Synthesis of the ionic dye, tris(2′,2 -bipyridyl) ruthenium(II) chloride ($Ru(bpy)_3^{2+}\cdot 2Cl^-$) within the supercages of a highly hydrophobic zeolite Y was carried out. Use of the neutral precursor $Ru(bpy)Cl_2(CO)_2$ allowed for high loading levels of $Ru(bpy)_3^{2+}$ (1 per 7 and 25 supercages). The emission quenching of the $Ru(bpy)_3^{2+}$-zeolite crystals dispersed in polydimethoxysiloxane (PDMS) films by dissolved oxygen in water was examined. The quenching data as a function of oxygen concentration was fit to a linear Stern-Volmer plot (R^2 = 0.98). Using the Stern–Volmer plot as calibration, changes in concentration of dissolved oxygen due to reaction with glucose in the presence of glucose oxidase was monitored. Human monocyte-derived macrophages internalized the submicron-sized $Ru(bpy)_3^{2+}$-zeolite crystals, and intracellular oxygen concentrations initiated by zymosan-mediated oxidative burst could be monitored by measuring the emission from $Ru(bpy)_3^{2+}$ by confocal fluorescence microscopy

Section 2: Publications/Presentations

Journal Articles

W.J.. Waldman, R. Kristovich, D. A. Knight, P. K. Dutta, [2007] Inflammatory properties of iron-containing carbon nanoparticles Chemical Research in Toxicology 20: 1149-1154.

Ruda-Eberenz, Toni A.; Nagy, Amber; Waldman, W. James; Dutta, Prabir K.. Entrapment of Ionic Tris(2,2'-Bipyridyl) Ruthenium(II) in Hydrophobic Siliceous Zeolite: O2 Sensing in Biological Environments, *Langmuir* (2008), **24(16)**, 9140-9147.

Schumacher, William; Nagy, Amber; Waldman, W. James; Dutta, Prabir K.. Direct Synthesis of Aqueous CdSe/ZnS-Based Quantum Dots Using Microwave Irradiation. *Journal of Physical Chemistry C* (2009), **113(28),** 12132-12139.

Peebles, Brian C.; Dutta, Prabir K.; Nagy, Amber; Waldman, W. James. Fenton Activity and Cytotoxicity Studies of Iron-Loaded Carbon Particles, Environmental Science and Technology, 2010, 44(17), 6887-92

Supriya Sabbani, Daniel Gallego , Amber Nagy, W. James Waldman, Derek Hansford, Prabir K. Dutta [2010] Synthesis of Silver-Zeolite Films on Micropatterned Porous Alumina and Its Application as an Antimicrobial Substrate, Microporous and Mesoporous Materials, (2010), 135(1-3), 131-136

Amber Nagy, Andrew Zane, Sara L. Cole, Michael Severance, Prabir K. Dutta and W. James Waldman, Biological Characterization and Internalization Mechanism of Microwave Synthesized Aqueous CdSe/ZnS Quantum Dots in Murine Macrophages, Chemical Research in Toxicology, submitted

Books

Dutta, P. K., Long, J. F., Williams, M. V., Waldman, W. J. Biological Activity of Mineral Fibers and Carbon Particulates: Implications for Nanoparticle Toxicity and the Role of Surface Chemistry, in Nanoscience and Nanotechnology, Ed. Vicki Grassian, 287-318, Wiley, NY (2008).

Graduate Student Dissertations and Presentations :

Dissertations
Amber Nagy : "Characterization and Interactions of Nanoparticles in Biological Systems"
Bill Schumacher : Microwave Synthesis of Quantum Dots
Brain Peebles : Correlations between surface structure and biological activity

Presentations:

Nagy, A., Zane, A., Dutta, P.K. and Waldman, W.J. “Biological Characterization of Synthesized Microwave-Irradiated CdSe/ZnS Quantum Dots.” Poster at the TechConnect World 2010 Conferences & Expo, hosting the Nanotech, Microtech, BioNano, Clean Technology and TechConnect Summit. Anaheim, CA. June, 2010.

Peebles, Brian C.; Nagy, Amber; Dutta, Prabir K.; Waldman, W. James. Pulmonary Toxicity of Manufactured Nanoparticles. Presentation, Central Regional Meeting of the American Chemical Society, Cleveland, OH, May 21, 2009.

Peebles, Brian C.; Nagy, Amber; Dutta, Prabir K.; Waldman, W. James. Pulmonary Toxicity of Manufactured Nanoparticles from the Perspective of Industrial Hygiene. Poster presentation, Ohio Nanotechnology Summit, Akron, OH, April 24-25, 2007.