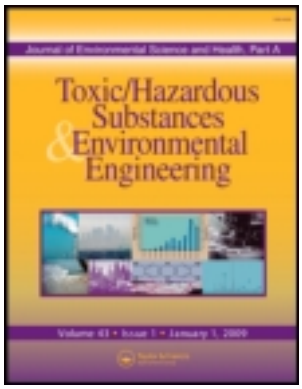

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Effects of Nanophase Materials (≤20 nm) on Biological Responses

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Effects of Nanophase Materials ($\leq$20 nm) on Biological Responses

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ABSTRACT

Nanophase materials have enhanced properties (thermal, mechanical, electrical, surface reactivity, etc.) not found in bulk materials. Intuitively, the enhancement of material properties could occur when the materials encounter biological specimens. Previous investigations of biological interactions with nanometer-scale materials have been very limited. With the ability to manipulate atoms and molecules, we now can create predefined nanostructures with unprecedented precision. In parallel with this development, improved understanding of the biological effects of the nanophase materials, whatever those may be, should also deserve attention. In this study, we have applied precision aerosol technology to investigate cellular response to nanoparticles. We used synthetic nanoparticles generated by an electrospray technique to produce nanoparticles in the size range of 8–13 nm with practically monodispersed aerosol particles and approximately the same number concentration. We report here on the potency of nano-metal particles with single or binary chemical components in eliciting interleukin-8 (IL-8) production from epithelial cell lines. For single-component nanoparticles, we found that nano-Cu particles were more potent in IL-8 production than nano-Ni and nano-V particles. However, the kinetics of IL-8 production by these three nanoparticles was different, the nano-Ni being the highest among the three. When sulfuric acid was introduced to form acidified nano-Ni particles, we found that the potency of such binary-component nanoparticles in eliciting IL-8 production

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was increased markedly, by about six times. However, the acidified binary nano-Na and -Mg nanoparticles did not exhibit the same effects as binary nano-Ni particles did. Since Ni, a transition metal, could induce free radicals on cell surfaces, while Na and Mg could not, the acidity might have enhanced the oxidative stress caused by radicals to the cells, leading to markedly higher IL-8 production. This result indicates the complexity of biological responses to nanoparticles. We believe that the exposure methodology and aerosol technology employed in our research will provide an effective means to systematically investigate cellular responses to nanoparticles, structured or unstructured, in ongoing research projects. Different cell lines, chemicals, and particle morphology can also be investigated using such a methodology.

Key Words: Nanophase metals; Nanoparticles; Electrospray; Differential mobility analyzer; Interleukenes; Cellular interaction.

INTRODUCTION

Materials research has traditionally focused on single atoms or on the bulk limit, but material properties can vary dramatically between these two extremes, where a few atoms bond together to form clusters and nanoparticles. The particles within this range are of increasing technological importance as semiconductors, biotechnology, and other fields employ nanotechnology in the 21st century. However, not all nanoparticles are created equal, and our understanding of the biological effects of nanoparticles is far from complete.

Unstructured ultrafine particles and nanoparticles are defined as aerosol particles produced without engineering control. We define ultrafine particles as particles $\leq$100 nm, and nanoparticles as those particles $\leq$20 nm. Particles emitted by internal combustion engines—such as engines used to power automobiles, trucks, and ships and to generate electricity—are examples of unstructured particles. Some fresh engine particles are ultrafine particles and nanoparticles. Diesel engine particles can be singlets and fractal aggregates of tens of nanometers such as those shown in Fig. 1. Traffic emissions represent a major contributor in today's metropolitan and urban pollution, with heavy-duty trucks the dominant contributor.

The biological effects of engine exhaust particles have been investigated extensively over the past several decades. For instance, the importance of diesel exhaust particles (DEP) in cytokine transcription has been documented.[1,2] Kawasaki et al.[3] showed that benzene-extracted components from DEP are responsible for cytokine production, oxidants-mediated NF-kappa B activation, and activation of the p38MAPK pathways in human bronchial epithelial cells. The U.S. Environmental Protection Agency (EPA) released a document in May 2002 indicating that DEP are a likely human carcinogen.[4] The vapor phase of diesel exhaust can also cause eye irritation, nausea, and respiratory problems. The EPA report, based on exposure to exhaust from diesel engines built before the mid-1990s, did not attempt to quantify the cancer risk. Data on biological responses to exhausts

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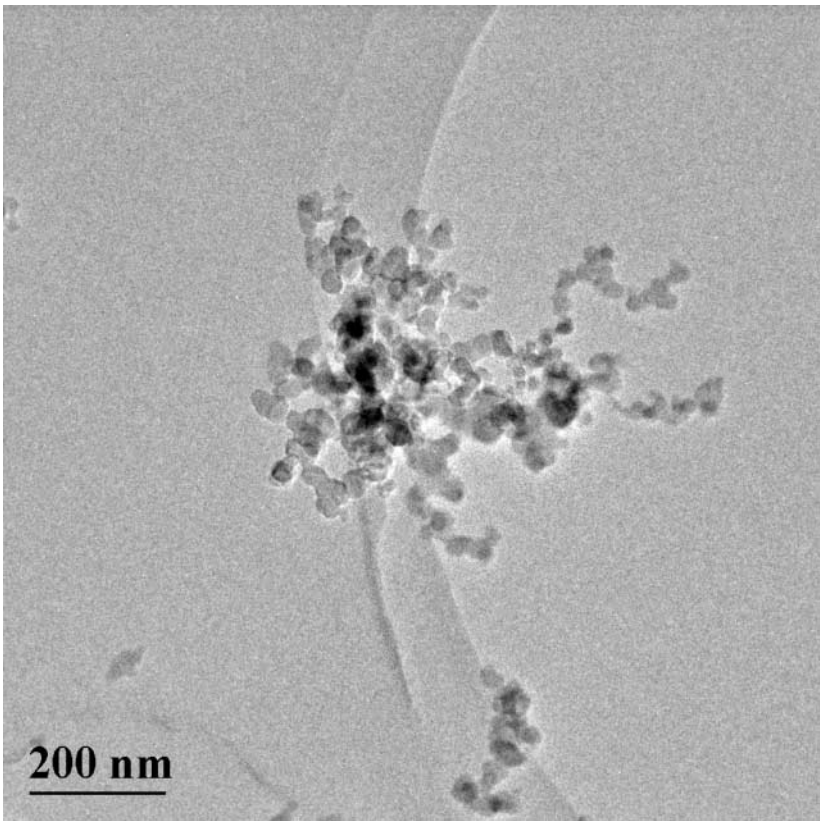

Figure 1. TEM photo of particles emitted by a diesel engine. Shown in the photo are aggregates of singlets forming a fractal-like structure.

from newer (after mid-1990s) diesel and gasoline engines that are significantly lower in particulate emissions are, however, very limited.

In the early 1990s, researchers at University of Rochester investigated the toxicity of very small particles. For instance, Ferin et al.[5,6] and Oberdorster et al.[7,8] showed that 20–30 nm TiO_2 particles cause pulmonary inflammation in laboratory animals, and death after 30 min of exposure to Teflon (or Polytetrafluoroethylene) particles in a similar size range. Rat cells did not respond to 1-μm TiO_2 particles in suspension,[9] a clear indication of enhancement of biological "reactivity" and/or "cytotoxicity" as the particle size decreased from the micrometer to the nanometer range for a given material. If the reduction in particle size from micrometers to nanometers could have such a profound effect on material properties, why would the same properties at the nanometer scale not have the equivalent effects on biological tissues as larger ones in the micrometer size range?

In fact, there is some experimental evidence in the literature to support this concern. For instance, healthy human subjects exposed to nano-Zn oxide particles (8–40 nm) showed significant increases in bronchoalveolar lavage (BAL) cytokine

production,[10] while no statistically significant responses (as compared to controls) were found when subjects were exposed to nano-Mg oxide particles.[11] Comparison of responses of rat lung cells to ultrafine Ni, Co, and TiO_2 particles of similar diameter indicates that ultrafine ($\leq$100 nm) Ni was much more potent than either Co or TiO_2 in causing inflammation,[12] although the effects of nano- vs. ultrafine particles were not separated in this study. However, the evidence was sufficient for the authors to suggest the potential role of free radicals produced by transition metals in inducing lung inflammation. At the nanometer scale, the number and the surface area of nanoparticles become a more important metric than mass. Surface area and numbers of ultrafine beryllium particles have been investigated as potential contributors to chronic beryllium disease.[13–15] Evidence indicating that surface area should be used as a metric for ultrafine particle toxicity was also reported by Brown et al.[16]

Industry is exploiting the potential and the profit margins of materials created from ultrafine particles and nanoparticles, called nanophase materials. For example, carbon nanotubes, which are highly structured nanoparticles, are being used because of exceptional material properties; a web site has a list of many companies that supply carbon nanotubes and related materials (http://www.personal.rdg.ac.uk/~scsharip/ tubes.htm). Yet this increased use of ultrafine and nanophase materials in a wide range of applications comes without our knowing exactly what the biological and environmental impacts of these materials might be. Thus, improving our understanding of biological responses to unstructured nanoparticles and structured nanophase materials is warranted. In this study, we report our findings on cellular responses to spherical nanoparticles, a special form of nanostructure, using a direct air-cell in vitro exposure technique that we have developed.[17]

MATERIALS AND METHODS

Generation of Nanoparticles for Testing

We produced nanoparticles of a narrow size distribution and consistent morphology using an electrospray apparatus from a number of prepared solutions. Particles produced by this technique are spherical. An example of the size distribution of particles made of $NiCl_2$ (prepared from electrospraying the $NiCl_2$ solution) is shown in Fig. 2. The size distribution was obtained by using a scanning mobility particle spectrometer (SMPS) equipped with a nano-differential mobility analyzer (TSI model 3936NL). As shown in Fig. 2, the peak diameter of the nano-Ni particles was 12.8 nm, and the geometric standard deviation (σ_g) characterizing the dispersion of the size distribution was 1.18. The shapes of size distributions for particles generated from other salts were similar to that in Fig. 2. The peak size (defined by the geometric mean diameter) and size dispersion (defined by the geometric standard deviation) were slightly different for different particles; the numeric values are shown in Table 1.

Solutions were prepared by dissolving predetermined amounts of salts into doubly deionized water. The solution was fed through a capillary of 25 μm inside

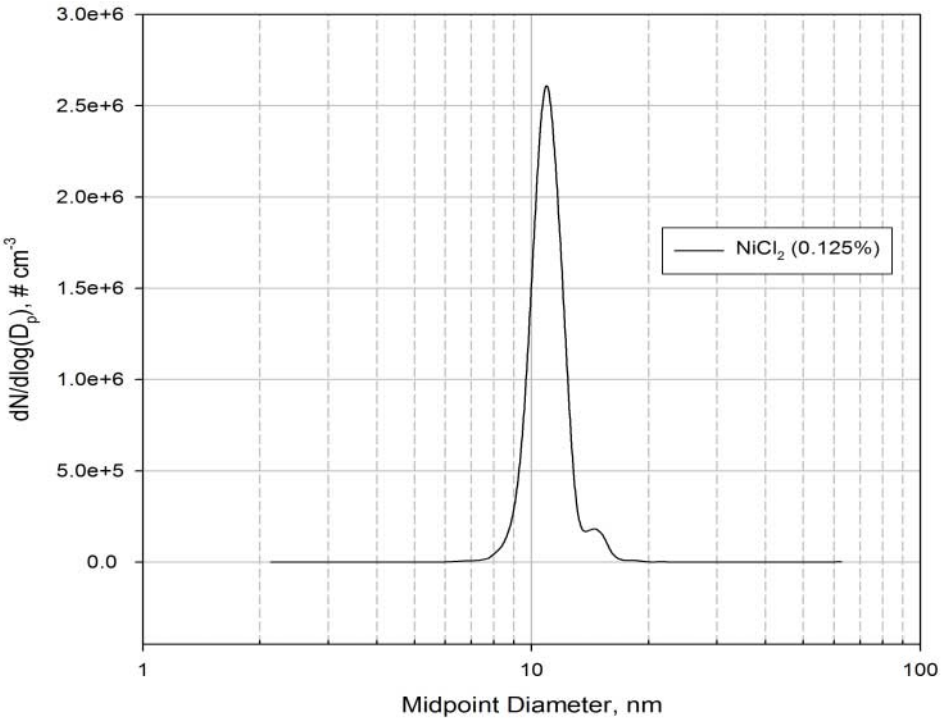

Figure 2. Example of particle size distribution produced by the electrospraying technique. Shown is the distribution of nano-Ni particles.

Table 1. Statistics for particle size distribution.

Main components	μ_g	σ_g	Nt	v (%)
Ni	12.8	1.13	5.7×10^5	7.3
V	10.8	1.15	5.3×10^5	9.4
Cu	9.5	1.15	5.8×10^5	7.2
A-Ni	11.5	1.17	5.9×10^5	7.5
A-Na	9.3	1.18	5.4×10^5	6.7
A-Mg	8.6	1.16	5.2×10^5	6.3

μ_g = Geometric mean diameter of a size distribution, nm, based on the number concentration.
σ_g = Geometric standard deviation based on the number concentration of a particle size distribution.
Nt = Total number concentration, $\#\,cm^{-3}$.
v = Variation of Nt throughout an experiment (%) computed as the ratio of standard deviation of Nt measured and the mean value of the Nt measured.
A-Element = Acidified-Element.

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diameter (ID). Depending on the operation, 2000–3000 V were applied to the exit of the capillary to enact the electrospraying process. A stable rate of nanoparticle generation was maintained when the electrospray was operated in the Taylor-cone mode. The output volumetric flow from this generator was approximately 1.5 ± 0.1 L pm, and the generation could be maintained stable for days using just a few milliliters of solution.

In the real world, particles are likely to have a mixed composition due to synthesis and/or contamination. We examined cellular response to mixed-composition particles by making particles of binary chemical composition. We mixed sulfuric acid with the solutions we used earlier in the electrospraying. The sulfuric acid solution was filtered twice (through a 0.4-μm filter) and mixed proportionally to a salt solution. For nano-Ni particles, the final solution was prepared so as to have mass concentrations of Ni (0.125%) similar to that of the solution used earlier. The nanoparticles produced by the electrospraying technique from the solution of sulfuric acid and metal salt would contain both species. The size statistics of these mixed particles are also shown in Table 1. Particle acidity is considered an important component in adverse health effects in humans because it impairs mucus clearance.[18]

The experimental setup for aerosol transport and the conditioning system is shown in Fig. 3. The generated particles were delivered into the manifold, in which dry High Efficiency Particulate Air (HEPA)-filtered air [relative humidity (rh) $\leq 6\%$] was added to the makeup of total flow needed for cell exposure and measurement of on-line aerosol size and composition. The manifold is made of PVC with a 10.16-cm ID. Several ports are installed on the manifold for sampling of (1) particles to cells

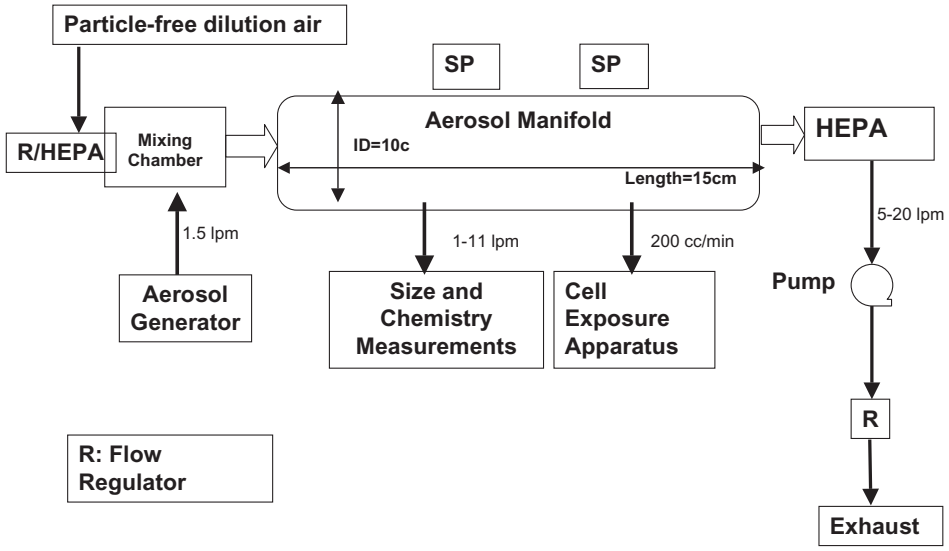

Figure 3. Experimental setup of aerosol transport and conditioning system used for cellular exposure.

and (2) particles to the SMPS and/or an aerosol-beam-focused laser-induced plasma spectrometer.[19] The aerosol plasma spectrometer provides a means for on-line monitoring of the composition of particles delivered to the cells, while the SMPS monitors the size distribution. The sampling ports are 30.5 cm apart, with the first port 30.5 cm from the entrance. The rh in the manifold was monitored on-line using an Omega rh and temperature probe and was maintained at 6–7%. The temperature in the manifold was 20–22°C. Under these conditions, we believe that the synthetic nanoparticles were solid. It is important to note that when particles were sampled into the cell exposure chamber (described in the next section), the particle flow was mixed with two separate flow streams before entering the chamber. One of the flows contained water vapor at $94 \pm 2\%$ rh, while the other contained $5 \pm 0.5\%$ CO_2 such that the atmospheric composition in the chamber was favorable to A549 cells.

Exposure Method

Assessing the biological effects of particles typically involves in vivo and/or in vitro instillation of an aqueous suspension containing collected airborne particles. That would require transfer of particles to a suspension. If the particles consist of dissolvable materials such as ammonium sulfate and nitrate, commonly found in the ambient air, a large fraction of the particle mass (60–70% on average for the airborne particulate matter observed in the southeastern United States) would be lost from the particle phase. Although the bias caused by the alternation of ionic strength, pH, and other properties of the aqueous phase would need to be assessed, more critically the physical, chemical, and biological properties of the target particles to be investigated could be significantly altered. Recently, alternative exposure techniques have been developed to enable in vitro toxicological study without the involvement of the aqueous phase.[20–22] The technique used in this study was described in Cheng et al.[17] and will be described briefly here.

We sampled aerosol particles into an array of cell exposure chambers at a volumetric flow rate of $200 \pm 15\,\text{mL}\,\text{min}^{-1}$ at room temperature. An array consists of six exposure chambers that are removed from an experiment at a preset time interval (e.g., 30 min, 1 h, 2 h, and so on). In other words, five exposure time points plus one reference sample of no particle exposure can be generated by this scheme. Alternatively, we can remove two chambers at a time at given points. In this experimental scheme, we can collect two time points (two chambers for no exposure, and four chambers for two time points).

A monolayer of A549 cells of approximately 8×10^5 counts was adhered to the top of a semipermeable polycarbonate transwell membrane insert of $1.0\,\text{cm}^2$ area. The pore size for a membrane is 0.4 μm. Each insert was placed inside a chamber; 1.5 mL of fresh medium was placed below, wetting the transwell membrane. Cells attached on the other side of the membrane are half exposed to the air (and particles during an experiment) and uptake the medium through the capillaries of the semipermeable membrane. We believe that in this way the properties of the aerosol

particles are preserved before they reach the cells. The possibility of altering particle properties was thus minimized.

Cell Model

In our study, the human type II lung epithelial cell line, A549, was obtained from ATCC (catalog number CCL-185) and subcultured according to ATCC's protocol. It is arguable why the type II cells were used, since cells are not the site of first contact for particles and will not be at the air interface for which the exposure technique is designed. The cells are actually below two complex layers of lipid and protein. The exposure method presumably can use any cells that can adhere to the membrane. Use of A549 cells as the model for studying cellular responses to air pollutants and/or other foreign chemicals has a long history, and a large database is available for us to reference and compare.

The A549 cells were cultured in a flask in F12K medium, Kaighn's modification, containing L-glutamine and sodium bicarbonate, 100 U each penicillin/streptomycin, 0.25–2.5 μg/mL Fungizone (if needed), and 10% fetal bovine serum in an incubator maintained at 5% CO_2, 37°C, and >94% rh conditions 48 h before an exposure experiment. For experiments simulating cells with preexisting illness conditions, the A549 cells were primed by adding 25 ng mL^{-1} of tumor necrosis factor (TNF)-α after 24 h of incubation. An additional 24-h incubation was conducted after the cell priming and before the exposure experiment (a procedure adapted from Stringer and Kobzik[23]). Standiford et al.[24] and Stringer and Kobzik[23] showed that use of A549 cells with and without the addition of TNF-α creates a model system that simulates the response of the normal lung and the lung with pre-existing inflammation when exposed to ultrafine particles.

Bioassay

In lung exposure to airborne particles, IL-8 has been identified as the key biomarker.[10,11,23–29] IL-8 is known to be a potent neutrophil chemotatic factor, a product of phagocytes and monocytes such as the A549 cells; the expression of IL-8 can be induced by inflammatory signals such as lipopolysaccharide, tumor necrosis factor, interleukin-1, and foreign agents such as aerosol particles. After exposure, cells on the transwell membranes were relocated overnight to a cluster plate in an incubator. Below the membrane was 3.1 mL of fresh medium. On the second day, the 1.5 mL of media from the exposure chamber and from the cluster plate were combined for enzyme-linked immunosorbant assay (ELISA) for IL-8 production using a commercial kit (OptEIA™ Human IL-8 Set kit from Pharmingen). Briefly, for ELISA, wells on a 96-well plate were coated with capture antibody, and supernatant was added. The IL-8 concentration was quantitatively determined, after incubation with a detection antibody conjugated with horseradish peroxidase, in comparison with a standard curve for IL-8. A log-log linear regression was used to establish the standard curve.

Data Analysis

Data analysis was performed using variances for multiple comparison tests and the linear regression method for calibration and interpolation calculations with Microsoft Excel, StatGraphics Plus, and SigmaPlot on a Pentium IV computer. Data uncertainty was estimated from each of six replicates of a concentration.

RESULTS

The geometric mean diameters of the nanoparticles, based on the number concentration, were between 8 and 13 nm (see Table 1). Since the geometric mean diameters of the test particles were similar and the number concentrations were close ($\leq \pm 5\%$) from one experiment to another, we think that particle size (and possibly surface area also) might not be a confounding factor in our study. Thus, we could safely deduce that the differences in IL-8 production might have resulted primarily from the differences in chemical composition.

Figure 4 shows IL-8 produced by unprimed A549 cells after being exposed to the nanoparticles for 2 and 4 h. The target components in separate experiments included V, Ni, and Cu. The mass concentrations of V, Ni, and Cu in solution were prepared also to be 0.125%. The same concentration was used for Na and Mg (Fig. 5) as well. Also shown is a comparison of cellular responses to exposure to single-chemical component nanoparticles of various compositions. Cell exposures to each element

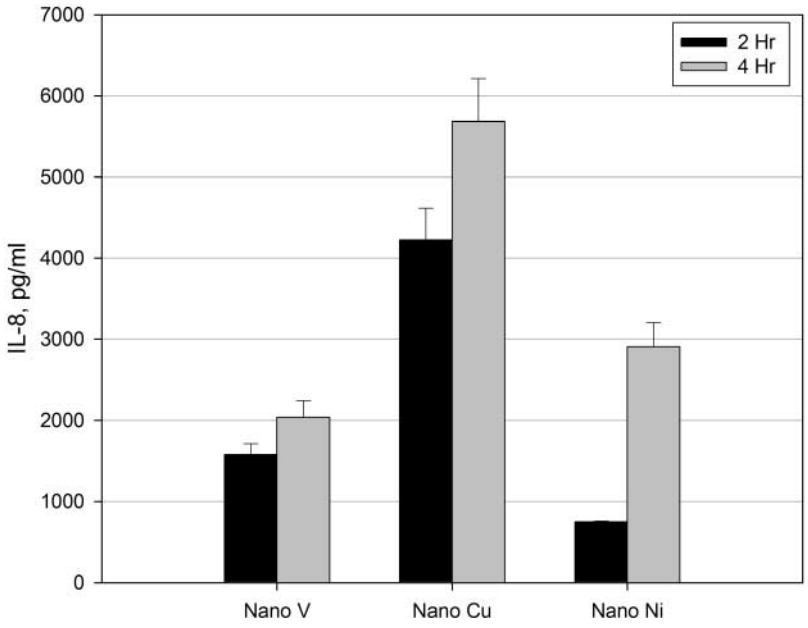

Figure 4. IL-8 production by A549 cells after exposure to three different nanoparticles for 2 and 4 h.

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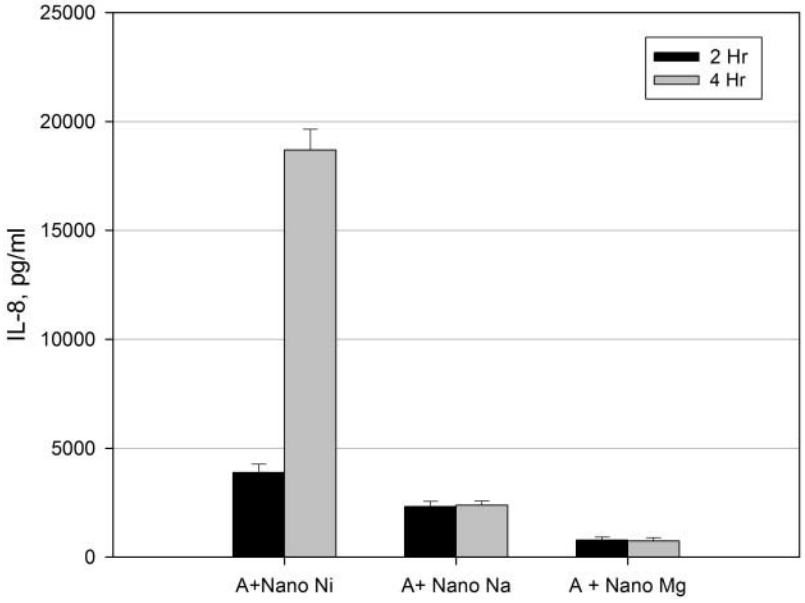

Figure 5. IL-8 production by A549 cells after exposure to three different binary-composition nanoparticles for 2 and 4 h.

were repeated twice on different days. As mentioned earlier, six replicas were analyzed for each sample. The error bar shown in Fig. 4 is one standard deviation of the 12 samples polled for an experiment.

Many other toxic metals (e.g., Hg, Cr, Cd, As, and Zn) are of potential interest to different people, but significant resources are required to do experiments on a long list of chemicals and combinations of them. Our choice of metals was based on our interest in other research work at Oak Ridge National Laboratory. The toxicity of nickel and copper has been investigated by others, and our results can add to the understanding of particle size effects. Vanadium is an important element in engine emissions, while magnesium and sodium are surrogate elements used in the aerosol production processes in our research on nanoparticle instrument development. Thus, from a worker safety point of view, the impacts of nanoparticles of these selected elements on humans are of interest.

The IL-8 produced generally increases as exposure time increases. Our earlier study indicated that IL-8 production began roughly 30 min after exposure started.[17] This result is consistent with the work of Kunkel et al.,[27] who showed that IL-8 production started 30 min after exposure and could last for 24–36 h after exposure ended. We observed that the increases of IL-8 with time are different for different nanoparticles, as shown in Fig. 4. Nanoparticles of nickel produced the most significant increase in IL-8 from 2 to 4 h; nano copper particles were next, while nano vanadium particles produced the least significant increase. This result indicates that while these three nanoparticles have similar toxicological effects on A549 cells, the kinetics of IL-8 production by chemically different nanoparticles is different.

In terms of the capability of producing IL-8 after a short-term exposure, nano-Cu particles appear to be more potent than the nano-Ni or nano-V particles.

The results of exposure to mixed-composition nanoparticles are shown in Fig. 5. It is obvious that the binary-component nano-Ni particles are much more potent in eliciting IL-8 production than the single-component nano-Ni particles. The kinetics of IL-8 production for the binary nano-Ni particles is similar to that of the single-component nano-Ni particles. On the other hand, nano-Na and nano-Mg particles do not exhibit similar potency in the presence of sulfuric acid. The IL-8 productions elicited by the acidified nano-Na or nano-Mg particles do not seem to follow the same kinetics that the nano-Ni particles do.

DISCUSSION AND CONCLUSIONS

Previous research on biological responses to nanoparticles (diameter < 10–20 nm) has been limited. Nanoparticles in this size region start to show unusual thermal, optical, electrical, and other physico-chemical properties due to quantum effects that one does not see in larger, micrometer or submicrometer size particles, as discussed earlier in this article. The interactions of these nanophase materials and engineered nanostructures (e.g., C_{60}, nanotubes, nanowires, etc.) with biological tissues and cells at the macro-scale remain to be explored. Therefore, use of these materials raises a concern about potential biological effects that we have yet to comprehend. With our ability to devise new methods and tools to manipulate atoms and molecules to make new materials of unprecedented precision, it would be wise for us as a society to gain deeper knowledge regarding the biological effects of nanophase materials and/or nanoparticles, the fundamental building blocks for nanotechnology. Similar cautions had been raised recently by Colvin[30] and Borm.[31] By understanding biological responses to nanoparticles, we may be able to use our new tools and abilities more carefully to produce products that are human and environmental friendly, and avoid environmental disasters such as those caused by DDT and Dioxin in the past.

In this study, we applied precision aerosol technology to the investigation of cellular response to nanoparticles. We used an electrospray technique to generate synthetic nanoparticles in the size range of 8–13 nm with practically monodisperse ($\sigma_g \leq 1.2$) aerosol particles of approximately the same number concentration ($\sim 5 \times 10^5\,\mathrm{cm}^{-3}$). The potency of nano-metal particles with single or binary chemical components in eliciting IL-8 production from the epithelial cell lines was investigated. For single-component nanoparticles, we found that nano-Cu particles were more potent than the nano-Ni and nano-V particles in producing IL-8. However, the kinetics of IL-8 production by these three nanoparticles was different; nickel nanoparticles have the fastest kinetics of the three. When sulfuric acid was introduced to form acidified nickel nanoparticles, we found that that the potency of such binary-component nanoparticles in eliciting IL-8 production was increased significantly, by about six times. However, the binary-Na and -Mg nanoparticles did not exhibit the same effects as that of binary nano-Ni particles on the cells. This result indicates the complexity of biological responses to nanoparticles. Nickel is a transition metal, while Na and Mg are not.

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Zhang et al.[12,32] showed that free radicals can be produced by transition metals on cell surfaces. The acidity of nanoparticles might have enhanced the oxidative stress caused by the presence of Ni; thus, the IL-8 produced was markedly higher than that produced by nano-Ni particles without the presence of sulfuric acid. We believe that the exposure methodology and aerosol technology employed herein will provide us an effective means to investigate cellular responses to nanoparticles, structured or unstructured, in the ongoing research projects. Different cell lines, chemicals, and particle morphology can be investigated using such an exposure methodology.

The data reported in this paper support the concerns regarding the potentially adverse impacts (to humans and possibly the environment) of nanoparticles, a fundamental building block in nanoscience and nanotechnology. The limited set of experiments that we conducted is in no way representative of the wide spectrum of chemicals and molecules that are being and will be exploited in nanotechnology applications. Many questions remain as to how other metals that were not investigated in our work could affect cellular responses. In addition to cellular responses, what are the physiological responses to nanoparticles? Furthermore, when the structure (e.g., a fractal aggregate instead of a single nanoparticle, a nanotube instead of a spherical particle, a bundle of nanowires or a single wire, etc.) and chemical composition of nanoparticles become more complicated and/or realistic, do we have a means to screen their biological impacts in a reasonable time? Do we have a system for categorizing nanoparticles as they are produced in large quantities in the future so that agencies such as the Occupational Health and Safety Administration and EPA can regulate them? The effects of nanoparticles vs. toxic gases if both are present will need to be investigated, since the physical dimensions of nanoparticles are now approaching those of large molecules and they behave aerodynamically similar to gas species. The presence of surface area of nanoparticles makes the biological responses to gas and to nanoparticles different. Do we have means to accurately calibrate nanoparticles in our biological investigation (i.e., a metrology issue)? How do we know we are measuring the right biological responses?

DISCLAIMER

Mention of the tradenames and instruments used in this article does not represent the endorsement of the author.

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