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Transcriptional responses of human aortic endothelial cells to nanoconstructs used in biomedical applications

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Abstract

Understanding the potential toxicities of manufactured nanoconstructs used for drug delivery and biomedical applications may help improve their safety. We sought to determine if surface modified silica nanoparticles and poly(amido amine) dendrimers elicit genotoxic responses on vascular endothelial cells. The nanoconstructs utilized in this study had distinct geometry (spheres vs. worms) and surface charge, which were used to evaluate the contributions of these parameters to any potential adverse effects of these materials. Time-dependent cytotoxicity was found for surfaced-functionalized but geometrically distinct silica materials while amine-terminated dendrimers displayed time-independent cytotoxicity and carboxylated dendrimers were nontoxic in our assays. Transcriptomic evaluation of HAEC responses indicated time-dependent gene induction following silica exposure, consisting of cell cycle gene repression and pro-inflammatory gene induction. However, the dendrimers did not induce genomic toxicity, despite displaying general cytotoxicity.

Keywords

silica nanoparticles; poly (amido amine) dendrimers; gene expression profile; endothelial cells

Introduction

Nanoscale particles and polymeric carriers are attractive platforms for drug delivery especially in anticancer therapy if strategies can be devised to take advantage of the enhanced permeability and retention (EPR) effect.^{1–4} Several carriers such as liposomes and albumin-based nanoparticles have been approved for use in the clinic.⁵ Other constructs

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Supporting Information Available.

Supporting Information includes tables of gene expression data and annotation analysis. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

such as poly(amido amine) (PAMAM) dendrimers⁶ and silica nanoparticles have shown promise as drug delivery platforms.^{7–11} Synthesis of dendrimers can be highly controlled to produce precise nanostructures with tailor-made surface groups to allow for conjugation of pharmaceutical and/or imaging moieties. SiO₂-based nanoparticles are being extensively studied for various medical applications due to the ease of synthesis and surface modification.⁷

The biomedical utility of nanoconstructs has great potential but safety concerns motivate toxicological study of the effects of particle size, shape, surface charge, agglomeration and surface contaminants on the physical and biochemical mechanisms that mediate effects on target organs.^{12–16} Indeed a recent publication indicates that occupational exposure poly acrylate-based nanoparticles has even resulted in death¹⁷ and there is suspicion that this may not be an isolated situation.¹⁸ While the dose of these exposures leading to pleuropulmonary disease is difficult to determine, it seems likely that these damaging occupational situations could be mitigated with appropriate personal safety equipment for employees. Nevertheless, traditional methods of evaluation of toxicity may have limitations in elucidating the mechanisms of action of these constructs. We hypothesize that toxicogenomic profiling of nanoparticle toxicity can complement traditional assays by identifying biomarkers that report exposure as well as identifying cellular signaling pathways that are affected by the nanoparticle.

Few investigators have studied the toxicity of a range of materials in a specific cell type that is likely to be exposed if the nanoparticles enter the blood circulation either deliberately or accidentally. Acute animal exposure studies show that nanoparticles transit through the vasculature and then can accumulate in the liver with poor renal excretion.¹⁹ In a previous study, we evaluated the acute *in vivo* toxicity of PAMAM dendrimers and silica nanoconstructs in mice.²⁰ In that study, amine-terminated dendrimers were 10× more lethal, based on administered dose, when compared to hydroxyl- or carboxyl-terminated dendrimers. In addition, the amine-terminated dendrimers promoted hemolysis as well as clotting of mouse and human blood samples. The introduction of silica nanoconstructs that were ~200 nm in diameter resulted in weight loss at ~30 mg/kg but silica spheres that were ~50 nm in diameter did not display overt toxicity except at very high doses (200 mg/kg); however a lethal toxicity was not observed until the very high doses, presumably due to pulmonary emboli.

In our current study, we evaluated the cytotoxicity of similar nanoscale platforms that varied by charge using carboxyl- and amine-terminated PAMAM dendrimers as well as geometry using amine-modified silica worms and spheres, on endothelial cells. Endothelial cells were chosen for evaluation since the *in vivo* acute toxicities were hematopoietic and vascular in our previous study.²⁰ Therefore, since these cells line the vasculature and interact with the clotting cascades and immune cells, we hypothesized that new toxicological mechanisms may reveal themselves in these cells. The amine-terminated dendrimers elicit a non-specific toxicity similar to the hemolysis previously observed in the blood samples and the silica nanoconstructs induce gene expression consistent with cell cycle arrest and pro-inflammatory gene induction.

Experimental Procedures

Cell culture

Primary human aortic endothelial cells were purchased from Lonza (Walkersville, MD) and were maintained in Medium 200 with supplements (Invitrogen, Carlsbad, CA). For experiments that used particulate matter, the nanomaterials were prepared as follows. The silica nanomaterials were washed with ethanol, dried and weighed. The weighed silica

powder was autoclaved dry and resuspended in a hood with sterile water to 25 mg/ml the material was vortexed and sonicated to resuspend the materials. Poly(amido amine) (PAMAM) dendrimers with distinct functionalization were purchased from Sigma (St. Louis, MO) and were further fractionated by a preparative Sephadex Hiload 75 size exclusion column (GE Healthcare Biosciences, Piscataway, NJ) as necessary to remove small molecular weight impurities. The dendrimers are suspended in deionized water at 10 mg/ml, vortexed, sonicated until the solution was clear, and then the solution was then filter sterilized prior to use.

WST-1 metabolic assay

The number of viable cells was determined using WST-1 assay which relies on tetrazolium salt reduction by NADH in live cells.²¹ Briefly, cells were seeded into 96 well plates at 20,000 cells/well for the 24 hr treatments and 5000 cells/well for the 72 hr treatments, and allowed to recover overnight. Toxicity of nanoparticles was evaluated at concentrations ranging from 1 – 500 µg/ml for the silica particles and between 0.1 – 100 µM for the dendrimers. Cells were treated as describe above (Cell culture) and their number was determined after 24 or 72 hrs. Following the nanomaterial treatment period, the WST-1 reagent was added and the absorbance at 460 nm and 650 nm was measured after incubation at 37°C for 1.5 hrs using a microplate reader.

Cell metabolism by ATP content

Cells were seeded into 384-well plates at densities of 1250, 2500, or 5000 cells/well in Medium 200 with supplements and allowed to adhere and recover. Nanosilica particles ranging from 10–1000 µg/ml were added and incubated with cells for 24 hrs, and viability was measured using CellTiter-Glo (Promega Corporation, Madison, WI) luminescent reagents as per the manufacturer's instructions. Luminescence was measured using a Perkin-Elmer VictorV³ Multimode Microplate Reader.

Characterization of the particulate matter

The nanomaterials used in this study were previously characterized in our previous studies (nano SiO₂²², dendrimers²⁰).

Microarray expression analysis

HAECs (6×10⁵ cells/well) were plated in 6 well plates and allowed to grow to >80% confluence and total RNA was collect after 4 and 24 hr exposure to the particulate matter. RNA concentration was determined with a Nanodrop spectrophotometer (Thermo Fisher, Wilmington, DE) and sufficient total RNA was recovered using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) protocol for the analysis. The quality of the RNA was monitored using an Experion automated electrophoresis station (BioRad Life Sciences, Hercules, CA) with standard sensitivity RNA chips (BioRad). Agilent labeling kits (Agilent Technologies, Inc., Santa Clara, CA) were utilized to amplify and generate Cy-dye labeled cRNA for hybridization to Agilent oligonucleotide arrays. A minimum of four biological replicates were collected representing each condition, and samples were combined, in equal fractions, for the microarray analysis. Three biological replicates were evaluated at each time point.

Agilent 44K (human whole genome) oligonucleotide microarrays were processed on site in the Microarray Resource located within the Huntsman Cancer Institute, University of Utah. Transcript levels were assessed on each channel and quantified by Agilent Feature Extraction software. This software preprocesses the data as follows. Local background is subtracted, irregular spots flagged, and global linear regression (lowess) normalization is performed, and this ratio is log transformed. The data were imported into TIGR MEV

software²³ for further analysis. Generally, a supervised strategy was used to identify the genes with the greatest significant differences between stimulated cells to unstimulated cells using multiclass SAM.²⁴ In each SAM analysis, 500 iterations were used when evaluating the false discovery rate, and a conservative cutoff was used that gave a mean false discovery rate of 0%. The gene expression profiles were hierarchically clustered for visualization. Gene Ontology, TRANSFAC and KEGG analysis of the differentially expressed genes was accomplished with GATHER.²⁵ An additional method of analysis was used to confirm our primary findings using Gene Set Enrichment Analysis (GSEA).²⁶

Cell cycle analysis

Cells were plated in 6 well plates and allowed to grow to >80% confluence similar to the gene expression studies. After 20–24 hr treatments with the nanomaterials, cells were trypsinized, washed with PBS, resuspended in NIM-DAPI, and incubated for 1 hr in the dark. Samples were analyzed using flow cytometry (Cell Lab Quanta SC, Beckman Coulter, Brea, CA) with a minimum of 20,000 events recorded for each sample. Cell cycle distributions were estimated using ModFit LT software Version 2.0 (Verity, Topsham, ME).

Immunoblot analysis

Cells were plated in 6 well plates and allowed to grow to >80% confluence as described above and then whole HAEC cell lysates were collected as described.²⁷ Protein concentrations determined by the Bradford method, the whole cell lysate was separated using NuPAGE 4–12% Bis-Tris gradient gels (Invitrogen Life Sciences, Carlsbad, CA) and transferred to a PVDF membrane (Millipore, Billerica, MA). The membrane was blocked in 5% milk in TBS-T and probed with antibodies directed at the gene products of BIRC3 (Epitomics, Burlingame, CA, S2700; 1:500), ZFP36 (Abcam, Cambridge, MA, ab36558; 1:250), PTGS2 (gift of Drs. Matthew Topham and Diana Stafforini, University of Utah; 1:200), MAP1LC3B (Sigma-Aldrich, St. Louis, MO, L7543; 1:1000), and GAPDH (Santa Cruz Biotechnology Inc., Santa Cruz, CA, FL-335; 1:1000). Secondary antibodies (donkey anti-rabbit, Calbiochem, San Diego, CA; donkey anti-mouse, Invitrogen, Carlsbad, CA) were routinely used at 1:5000 dilutions. Protein was detected using chemiluminescence and visualized on a Kodak ImageStation 440.

Statistical Analysis

The analysis of the microarrays is presented above. Other data are presented as mean $\pm$ standard deviation. EC₅₀ values were derived by fitting the data with logistic curves using four parameter fitting using Origin version 7.5 (OriginLab, Northampton, MA). Two-way ANOVA with Holm-Sidak post-hoc analysis was used to evaluate the cell cycle analysis (SigmaStat Version 3.5, Aspire Software International, Ashburn, VA), $p < 0.05$ was considered significant.

Results

Cell Viability

The safety of nanoparticle platforms for medical applications is paramount to their broad utility. Vascular exposure is likely to occur if nanoconstructs are used for diagnostics, therapeutics, or theranostics. However only a limited number of nanoparticles have been evaluated for their interactions with endothelial cells in detail.^{28–34} These studies suggest that nanoparticles, from carbon nanotubes to quantum dots display dose-dependent cytotoxicity. The nanoparticles used in this study have been previously characterized and their cytotoxicity evaluated in mouse macrophages (RAW 264.7 cells), human lung adenocarcinoma epithelial cells (A549), and colon carcinoma cells (CaCo-2).^{22, 35–37} Our

first goal of this study was to expand the evaluation of these nanoconstructs to primary human endothelial cells (HAECs) consistent with exposure if used in medical applications where they would be carried in the blood. These assays were designed to determine if there was a concentration, cell density, and time dependence to the cytotoxicity responses of HAECs to the silica particles and dendrimers. HAECs were treated for 24 (cells plated at 20,000 cells/well) or 72 hrs (cells plated at 5000 cells/well) with increasing doses of silica nanoparticles or the generation 3.5 or 4 PAMAM dendrimers (G3.5-COOH or G4-NH₂, respectively) and cellular metabolism, as a measure of cell viability, was assessed using the WST-1 formazan assay in 96 well plates. The G3.5-COOH dendrimers did not demonstrate any appreciable cytotoxic activity up to 50 μ M. In comparison, the G4-NH₂ dendrimers showed considerable cytotoxicity with little time or cell plating dependence; the EC₅₀ at 24 hrs was 2.2 ± 0.1 μ M and at 72 hrs was 1.6 ± 0.1 μ M (Figure 1A). The results for the G4-NH₂ dendrimers were similar to previous assessments in other cell types.³⁶ The cytotoxicity of the G4-NH₂ dendrimers appeared analogous to the hemolysis we previously reported²⁰ and similar to the reported disruption of membranes from these positively charged nanoparticles.^{38–40}

Both the spherical and worm-like silica nanoparticles demonstrated similar overall dose-dependent cytotoxicity in HAECs. The silica nanoparticle spheres were more toxic and the EC₅₀ at 24 hrs (cells plated at 20,000 cells/well) was 43 ± 7 μ g/ml that decreased to 5.5 ± 0.2 μ g/ml at 72 hrs (cells plated at 5000 cells/well). The worms displayed only modest cytotoxicity with an EC₅₀ at 24 hrs of 116 ± 6 μ g/ml but EC₅₀ value dramatically decreased to 6 ± 4 μ g/ml at 72 hrs (Figure 1B). These differences in EC₅₀ could be due to a number of variables; a cell density dependence cytotoxicity could be an explanation and the WST assay might be sensitive to the silica nanoconstructs. Therefore, an additional viability assay was also used to verify the formazan-based assay and test if the toxicity of nanoparticles was dependent on cellular confluency. This assay was performed in 384 well plates and ATP content was measured through a luminescent assay (CellTiter GLO). Distinct numbers of cells were seeded to generate different levels of confluency with an overnight incubation from 5000, 2500, and 1250 cells/well to represent ~100%, ~75% and ~50% confluence at the time of treatment with silica nanoparticles for 24 hrs. The ATP assay generated similar results to the WST-1 assay and demonstrated a seeding-density response (Figure 2). The cytotoxicity response suggests that the amount of silica nanoparticles/plate surface area may be more appropriate when considering the cellular responses to these materials in vitro.

Gene Expression

Gene expression analysis was carried out using Agilent Whole Genome Human microarrays. HAECs were treated in 6 well plates where they were allowed to grow to >80% confluency through daily media exchange. The doses of the nanoconstructs were chosen such that the cells demonstrated little or no overt cytotoxicity (>90% viable) within the time-frame of the gene expression analysis. The cells were treated with PAMAM dendrimers at 0.5 μ M for 4 and 24 hrs. The dose for the PAMAM dendrimers was below the EC₅₀ for the amine-terminated dendrimers since no time dependence was observed, based on cytotoxicity, and we wanted to assess live cells for the transcriptional analysis. The silica nanoparticles were used at 100 μ g/ml (35 μ g/cm²) for 4 hrs for both the worms and spheres, and at 24 hrs for the worms. The spheres were also evaluated at 73.4 μ g/ml (~25.7 μ g/cm²) for 4 and 24 hrs to adjust for surface area differences between the worms and spheres. The rationale for the different doses for the worms and spheres was to maintain constant silica surface area since previous work by others in mouse macrophages and found, when normalized to surface area of the nanoparticles, similar responses among the silica nanoparticles they evaluated.⁴¹ The doses used for the silica particles were above their respective EC₅₀ for 72 hrs of exposure; however no overt cytotoxicity was observed within 24 hrs for these nanoconstructs.

Therefore, the gene expression analysis might provide more insight into the cytotoxic mechanisms of the silica nanoconstructs. The confluence of the cells and the morphology of the HAECs incubated with nanoparticle platforms were visually inspected prior to the collection of RNA. Modest cytotoxicity of some treatments was apparent with cells becoming more refractive and more cell-free space was observed on the plates. The silica nanoparticles, particularly the worm geometry, displayed intracellular vesicle formation while the G4-NH₂ dendrimers appeared to promote disruption of the cell monolayer. Nevertheless, high quality RNA (RNA quality indices were >7) was collected from all treatments and samples from each treatment were competitively hybridized with RNA samples derived from untreated HAEC to phenotypically anchor all the gene expression data.

Utilizing multi-class Statistical Analysis of Microarrays (SAM) to evaluate the different nanoparticles as well as the distinct time points it was clear that incubation with the silica nanoparticles promoted broad transcriptional responses that demonstrated time-dependence. Incubation with the G4-NH₂ dendrimers did not show time-dependent responses, and the G3.5-COOH dendrimers did not elicit a consistent transcriptional response (Figure 3). This analysis identified genes that were modulated by the nanoconstructs. An analysis of the gene annotation using the software, Gene Annotation Tool to Help Explain Relationships (GATHER), to evaluate the KEGG pathway database, the genes demonstrate significant enrichment (Bayes factors of 16.6 and 13.4, respectively) of two primary pathways; cell cycle (path:hsa04110) and cytokine-cytokine receptor interactions (path:hsa04060). GATHER was also used to identify statistically enriched Gene Ontology (GO) categories with multiple levels represented including; cell cycle, pro-inflammatory genes, and sterol biosynthesis (see supplementary tables). The induced genes were statistically enriched for pro-inflammatory genes. The genes that were depressed were statistically enriched for cell cycle (particularly genes involved in progressing through mitosis), and sterol biosynthesis.

The data was re-analyzed using two-class unpaired SAM to compare all silica nanoparticle samples to the G3.5-COOH dendrimers to identify the explicit silica-dependent gene modulation. The G3.5-COOH dendrimers did not elicit a transcriptional response and so could serve as controls (Figure 4). This analysis identified 289 unique genes where annotation analysis using GATHER identified wound healing and other pro-inflammatory gene ontology categories as prominent transcriptional responses. Gene ontology categorization suggests that genes that were induced primarily represent proinflammatory genes (including genes regulated by NF- κ B when assessed for enrichment using GATHER to evaluate gene annotation with TRANSFAC) while genes involved in sterol biosynthesis and progression through G2/M phases of the cell cycle were suppressed (see supplementary tables).

There were few genes that clearly distinguished the silica worms from the silica spheres but there was a pronounced time dependence of gene expression alterations following silica nanoparticle exposure (Figure 5). Many of these genes were cytokines and histones. These gene expression changes were consistent with alterations in the regulation of cell cycle genes as well as pro-inflammatory genes (see supplementary tables).

Since microarray data can have a low signal to noise ratio, an additional approach was used to determine the robustness of our interpretation. The gene expression data was re-evaluated using GSEA. This method rank orders the expression profiles and then analyzes the ranks to determine enrichment of genes, particularly those represented as high expressers or low expressers. A 'difference of classes' analytical approach was used to discriminate gene enrichment among treatment categories, that is, G3.5-COOH dendrimer treatments compared to either silica nanoparticles or G4-NH₂ dendrimers. With this approach we

identified gene sets enriched in this dataset that have been identified in curated gene sets in the Molecular Signatures Databases including; chemical and genetic manipulation datasets, BioCarta datasets, KEGG datasets, and the molecular functions dataset from the Gene Ontology database. With gene set size filters of a minimum 15 genes or a maximum of 500 genes, we evaluated 2398 gene sets in the database, and we found statistical enrichment 25 datasets (FDR q-values <0.25). The gene sets with lowest q-values demonstrated decreased expression in silica nanoparticle treatment. The most enriched gene sets were genes suppressed and included genes involved in cell cycle progression, particularly mitosis as well as sterol biosynthesis (Figure 6A, B). The most enriched gene set that represented induced gene expression were pro-inflammatory genes consistent with NF- κ B-dependent gene induction (Figure 6C). In the G3.5-COOH v. G4-NH₂ comparison, genes were suppressed that were involved in sterol biosynthesis. Since these genes were also identified with the silica particles, these results suggest that the positively charged surface of these nanoparticles elicit this response independent of the nanoparticle type. In addition, metabolomic experiments to evaluate sterol biosynthetic pathways did not provide additional evidence of imbalanced sterol intermediates (data not shown) suggesting that these changes in gene expression may be early signs of cell death associated with decreased lipogenesis.

Previous genomic studies with engineered silica spheres of different sizes ranging from 10 to 500 nm in diameter displayed particle surface area-dependent cytotoxicity, and gene expression were performed in murine macrophages.⁴¹ All of these materials had similar negative surface charge (overall negative zeta potential) which is distinct from the positive surface chemistry silica nanoparticles used in these experiments. Nevertheless, the silica nanomaterial study in murine macrophages, with distinct sizes, surface chemistry, and geometry induced a similar set of 31 genes to the genes induced in this study, and these genes are most consistent with early stress-response and pro-inflammatory genes (TNF, ATF3, GEM, CXCL2, PTGS2, CCL2, FOS, CCL4, MTMR4, HIST1H2BC, EGR2, NR4A1, HMOX1, KLF4, GADD45B, GDF15, MAFF, EGR1, BCL2L11, PDE4B, JUNB, ZFP36, SLC25A25, DUSP1, CDKN1A, BBC3, ABCA1, RAB20, KLF2, TYMS, and TXNIP). This overlap of gene expression changes resulting from distinct geometry and surface chemistry may suggest a relatively conserved cellular response to silica nanoparticles. One possibility is that the cells are responding to these silica nanoparticle platforms as if they were pathogens.^{42–44}

Overall, the dendrimers displayed few responses that provided insight into potential mechanisms of toxicity. The G3.5-COOH dendrimers showed little toxicity as well as little transcriptional alterations. The G4-NH₂ dendrimers showed concentration-dependent cytotoxicity consistent with previous reports.^{36, 37, 45, 46} The dendrimers toxicity to HAECs and the transcriptional data do not display time-dependence and therefore it seems unlikely that any transcriptional change is related to the cytotoxic mechanism of this nanoconstruct. Nevertheless, to our knowledge, this is the first evaluation of cytotoxicity and whole genome transcriptional responses of multiple engineered nanoparticle species that differ in surface charge (PAMAM dendrimers) or distinct geometries (silica spheres and worms) in endothelial cells.

We sought to validate the interpretation of the silica-mediated transcriptional alterations. HAECs were plated and grown to >80% confluency and then treated with nanoparticles just as in the gene expression experiments (0.5 μ M for the dendrimers, 100 μ g/ml (35 μ g/cm²) for the silica worms, and 73.4 μ g/ml (~25.7 μ g/cm²) for the silica spheres). After 20 hrs, the cells were evaluated for DNA content. The control cells and the dendrimer-treated (both G3.5-COOH and G4-NH₂) cells generated similar cell cycle profiles but the silica (both spheres and worms) treated cells displayed statistically significant decreases in G2/M as

predicted by the gene expression alterations as well as the compensatory changes in G0/G1 (Figure 7) validating the suppression of mitotic genes identified by both SAM and GSEA.

Protein levels of genes that were induced were evaluated as additional validation of the transcriptional changes observed. Therefore, several pro-inflammatory as well as other gene products related to cellular interactions with nanoparticles were evaluated by immunoblot analysis and confirmed the induction by the silica nanoparticles (Figure 8). Since many of the genes displayed silica-induced expression by the 4 hr time point, the protein levels were evaluated at 4 and 8 hrs. The dose of silica nanoparticles was also evaluated at the dose used for gene expression profiling (worms, 35 $\mu\text{g}/\text{cm}^2$, and spheres, 25.7 $\mu\text{g}/\text{cm}^2$) and at half that dose to determine if the cells responded at more modest doses. Gene products for BIRC3, PTGS2 and ZFP36 all displayed time- and dose-dependent protein induction consistent with the induction of pro-inflammatory genes regulated by NF- κ B. Nanoparticles and NF- κ B family members have been associated with the modulation of autophagy and LC3B protein cleavage.^{47–52} Therefore, we selectively evaluated the gene expression of the autophagic gene, MAP1LC3B, and its gene product LC3B. A modest increase in RNA expression and a more robust increase in protein expression as well as processing of LC3B were observed in cells incubated with the silica materials but not the dendrimers (Figure 8). Again, these similarities could reflect a cellular response to these nanoparticles similar to pathogen-mediated responses.

Discussion

Our previous cell-based studies have focused on PAMAM dendrimer permeability across epithelial cell barriers^{37, 45, 53} or interactions of silica with macrophage- or epithelial-like cells.^{22, 54} *In vivo* studies have demonstrated vascular and hemolytic toxicities associated with both of these classes of nanoconstructs.^{20, 55} Therefore, studies suggest that some nanoparticles may exacerbate hematological/vascular disease states should their concentrations become high enough in these cells. Our current studies in endothelial cells suggest that silica, at $\sim 20 \mu\text{g}/\text{cm}^2$ doses induces pro-inflammatory and cell cycle arrest responses. G4-NH₂ dendrimers also displayed cytotoxicity but without a similar induction of pro-inflammatory genes. The dendrimers toxicity to HAECs and the transcriptional data do not display time-dependence and therefore it seems unlikely that any transcriptional change is related to the cytotoxic mechanism of this nanoconstruct. This is the first evaluation of cytotoxicity and whole genome transcriptional responses of multiple engineered nanoparticle species that differ in surface charge (PAMAM dendrimers) or distinct geometries (silica spheres and worms) in endothelial cells.

Toxicogenomic studies provide an unbiased and global assessment of cellular or tissue responses to interactions with nanoparticles and the biological responses to these materials may be based on their physicochemical properties. A previous study evaluated the size dependence of gene expression following exposure to silica nanoparticle spheres and demonstrated that the nanoparticle's surface area was the major influence rather than the diameter of the nanoparticle per se.⁴¹ In the current studies, we have observed gene expression responses to silica nanoparticles that overlap considerably with that study. However, the responses to silica nanoparticles are distinct from other nanoparticles. For example, our previous studies with zinc oxide (ZnO) demonstrate considerable cytotoxicity,⁵⁶ and causes numerous gene expression changes resulting from Zn²⁺ dissolution even at modest *in vitro* exposures.⁵⁷ Nano-sized ZnO is being used in topical lotions, cosmetics, and sunscreens which are clearly distinct applications compared to the potential for silica nanoparticles and dendrimers to serve as platforms for use in nanomedicine. The core nanoparticle distinctions in mechanism of action are easily differentiated with gene expression profiling.

This study has provided some of the first data on the effects of nanoparticle constructs on human endothelial cells. The silica nanoconstructs elicited genes that regulate cell cycle arrest and a pro-inflammatory response in HAECs. The precautionary principle supports continued research on the health effects of new industrial chemical formulations, including smaller primary particle sizes and novel surface treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

BIRC3	baculoviral IAP repeat containing 3 (also known as cIAP2, HGNC ID=591)
GAPDH	glyceraldehydes 3-phosphosphate dehydrogenase (HGNC ID=4141)
GATHER	Gene Annotation Tool to Help Explain Relationships
GSEA	Gene Set Enrichment Analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
MAP1LC3B	microtubule-associated protein 1 light chain 3 beta (also known as LC3B; HGNC ID=13352)
PTGS2	prostaglandin-endoperoxide synthase 2 (also known as COX-2, cyclooxygenase-2, HGNC ID=9605)
TBS-T	Tris buffered saline with 0.1% Tween-20
TIGR MEV	the institute for genomic research multiple experiment viewer
TNF	tumor necrosis factor (HGNC ID=11892)
TRANSFAC	gene regulation transcription factor database
SAM	significance analysis of microarrays
SiO₂	silicon dioxide
ZFP36	zinc finger protein 36 (also known as TTP, tristetraprolin, HGNC ID=12862)

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Figure 1.

Effects of nanoparticles on metabolic activity of HAEC, as determined by the conversion of MTS to formazan. A) Dendrimer (G3.5-COOH, upward pointing triangles, and G4-NH₂, downward pointing triangles); B) Silica nanoparticles (spheres, circles, and worms, squares). Cells were incubated with nanoparticles for 24 or 72 hrs.

Figure 2.

Effects of silica nanospheres (A) and silica nanoworms (B) on metabolic activity of HAEC, as determined by measuring ATP concentration. HAEC were plated in 384-well plates at initial density of 5000 cells/well (squares), 2500 cells/well (circles), and 1250 cells/well (triangles). Cells were incubated with nanoparticles for 24 hrs. The bottom axis represents the dose of silica nanoparticles in $\mu g/ml$ while the top axis represents the dose of silica nanoparticles in $\mu g/cm^2$.

Figure 3.

Gene expression responses when comparing all groups (silica worms (W, 35 $\mu\text{g}/\text{cm}^2$), spheres (S, 35 $\mu\text{g}/\text{cm}^2$, or surface area adjusted Ss, 25.7 $\mu\text{g}/\text{cm}^2$), dendrimers G3.5-COOH (G3.5C, 0.5 μM) and G4-NH₂ (G4N, 0.4 μM) at 4 and 24 hrs (one additional sample of worms at 1.5 hr. A) Sample tree (a, b, and c refer to independent biological triplicates) and the continuous color gene expression scale from green (repressed compared to the untreated control) to red (induced compared to the untreated control). B) Heat map of gene expression from multiclass Significance Analysis of Microarrays. C) Top ten gene ontology categories with levels of 4 or greater. The size of the wedge is representative of the number of genes representing the category while the numbers represent the Bayes factor. Six of the top ten

categories related to cell cycle and the other four related to genes involved in inflammatory responses.

Figure 4.

Gene expression responses when comparing G3.5-COOH dendrimers as a control compared to the silica treatments (worms (W) and spheres (S and Ss) at the same doses used in Figure 3). A) Sample tree and gene expression scale (as described in figure 4). B) Heat map of gene expression from two class Significance Analysis of Microarrays comparisons between the G3.5-COOH dendrimers and silica nanoparticles. C) Top ten gene ontology categories at GO levels of 4 or greater. As in figure 3, the size of the wedge is representative of the number of genes representing the category while the numbers represent the Bayes factor.

Figure 5. Time-dependent responses for silica nanoparticles when comparing the 4 hr time point v. the 24 hr time point. A) Sample tree and gene expression scale (as described in figure 3). B) Heat map of gene expression from two class Significance Analysis of Microarrays comparisons between the 4 hr and 24 hr silica nanoparticles. C) Top ten gene ontology categories with levels of 4 or greater. As in figure 3, the size of the wedge is representative of the number of genes representing the category while the numbers represent the Bayes factor.

Figure 6.

Gene Set Enrichment Analysis profiles identifying gene sets that were similar to those enriched in this study. A) Inverse correlation of cell cycle gene expression, particularly genes involved in the G2/M transition. B) Decreased expression of sterol biosynthetic genes associated with positive charged nanoparticles. C) Induced expression of pro-inflammatory consistent with NF- κ B-mediated gene induction.

Figure 7.

DNA content analysis of HAECs after 20 hrs treatment with nanoparticles. Similar data was obtained for both types of dendrimers (G3.5-COOH and G4-NH₂) and both geometries of silica (worms and spheres) so all of the data was combined for 6 replicates of control, dendrimers and silica. Asterisks indicate statistically significant ($p < 0.05$) difference with controls (untreated cells).

Figure 8.

Immunoblot validation of gene expression changes from whole cell lysates. Time and dose (for silica particles but not dendrimers) evaluation of protein expression by immunoblot analysis following stimulation of HAECs with nanoparticle platforms of the gene products of BIRC3, PTGS2, ZFP36, MAP1LC3B, as well as GAPDH as a loading control. Ten micrograms of total protein were loaded in each lane. No changes in protein expression were observed in dendrimer treatments but time- and dose-dependent increases in protein expression following silica stimulation were observed that validate the microarray gene expression observations. The lower panel shows quantitation of repeated immunoblots.