One low-dose exposure of gold nanoparticles induces long-term changes in human cells

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We report the in vitro long-term (20 wk) changes in cells exposed to well-characterized gold nanoparticles (Au NPs) with varying shapes and surface coatings under both chronic (exposure to Au NPs continuously over 20 wk) and nonchronic (initial acute cell exposure to Au NPs, followed by 20 wk in NP-free cell media) conditions. Both chronic and nonchronic Au NPs exposures at low dose induce modifications at the gene level after long periods. In attempt to overcome from the injuries caused by nanoparticle exposure, genes related to oxidative stress, cell cycle regulation, and inflammation are among those presenting differential expression levels. Surprisingly, the nonchronic exposure induced more gene expression changes than its chronic counterpart and the stress effects caused by this type of exposure were sustained even after 20 wk without any additional NP exposure. NP surface chemistry played an important role in the alteration of gene regulation. Overall, our data suggest that (*i*) cells can adaptively respond to chronic, low-level NP insults; (*ii*) the cell stress response is not reversible over time upon removal of NPs upon acute, nonchronic exposure; and (*iii*) polyethylene glycol is not as benign a surface chemistry as is generally supposed.

gold nanoparticles | acute exposure | chronic exposure | surface chemistry | gene expression

Gold nanoparticles (Au NPs) are promising materials in biomedicine (1, 2). A combination of extraordinary factors (tunable optical absorption and scattering, photothermal and photoacoustic efficiencies, tunable size/shape/surface chemistry, and lack of toxicity in the bulk) make them ideal for biological applications that range from sensing, to targeted drug delivery and therapy, to diagnostic imaging (1, 2). Moreover, the presence of Au NPs in consumer products such as cosmetics is growing over time (3). With the increased exposure of engineered NPs to biological systems, regulatory agencies are raising concerns regarding NP adverse effects on human health and the environment (4, 5). There are considerations unique to NP cell/body interactions (as opposed to molecules) that need to be thoroughly understood for safe use of nanoscale materials (4, 5). The same multivalent properties that allow for nanoscale display of targeting surface moieties and access to the inside of cells make cell–NP interactions more difficult to predict. In addition to the size, shape and composition of NPs themselves, the interactions between their organic surface coatings and biological environments must be analyzed, and other variables such as aggregation state must also be considered.

Any observed cytotoxicity of Au NPs depends on physicochemical properties such as size and surface chemistry. A majority of toxicity studies have found Au NPs to be mostly nontoxic after acute exposures, as long as the particles are above 4–5 nm in diameter (below this size, Au NPs become catalytically active and can induce cytotoxicity) (4, 6, 7). Au NPs larger than 5 nm that cause toxic effects to cells often have inherently toxic surface coatings (6). Many other findings of acute toxicity can be attributed to the use of very high concentrations and different cell type sensitivities (7–10). The NP physiochemical properties also affect the ability of the cell to uptake NPs, which itself can influence toxicity (11). Most of the literature available on the toxicological impact of Au NPs focuses on whether there is an acute stress response (viability, oxidative stress, or apoptosis). However, cells exposed to subcytotoxic doses of different Au NPs have been shown to undergo morphological changes accompanied by F-actin disruption in human dermal fibroblasts (12). Others have found low doses of Au NPs to alter cell migration and proliferation, stem cell differentiation, and membrane receptor internalization (13–16). Many chemicals that trigger changes to cellular signaling pathways and induce molecular responses elicit no acute toxicological response (17). Therefore, a more nuanced description of NP effects beyond "toxic" or "not toxic" is needed.

The surface chemistry of Au NPs most likely plays the largest role in determining the interactions of Au NPs with biomolecules. High local concentrations of charged ligands on NP surfaces may interact with cell receptors differently than the ligands would alone, resulting in unexpected cell signaling changes (18). It is also well known that NPs bind free molecules in the biological milieu, resulting in protein coronas whose compositions depend on the identity, density, lability, hydrophobicity, and charge of the surface ligands (19, 20); this corona formation may decrease the availability of certain biomolecules by capturing (adherence) and/or inducing structural changes that could lead to alteration of cell signaling pathways (21–23). Size and shape may affect these interactions as well by influencing both uptake rates and contact area between biomolecules and NP surfaces (7, 11, 18). A reliable and quantitative way to measure effects of NP shape, size, and surface coating on cell signaling and biomolecule availability is to perform gene expression profiling. Analysis of gene expression can determine which cellular pathways are affected by NP exposure to elucidate subtle cellular changes and provide mechanistic insight into NP–cell interactions at the molecular level. Various types of Au NPs have been tested in vitro at the gene expression level after acute exposure: coatings range from citrate to charged polyelectrolytes to poly(ethylene glycol) (PEG), particle concentrations

Significance

Gold nanoparticles are promising candidates for optical sensing, bioimaging, delivery, and therapeutic applications due to their size- and shape-dependent physical properties and inherent biocompatibility compared with other metallic nanoparticles. Many studies have found that these particles are generally nontoxic at low doses for most, but not all, initial surface treatments. In this study, a long-term (20 wk) exposure of a set of gold nanoparticles to a human cell line was performed in two ways: an acute burst of nanoparticle exposure compared with a continuous, chronic exposure at the same concentration. Overall, it was found that an acute burst of exposure is more harmful to cells, and that cells can adapt to long-term nanoparticle exposure.

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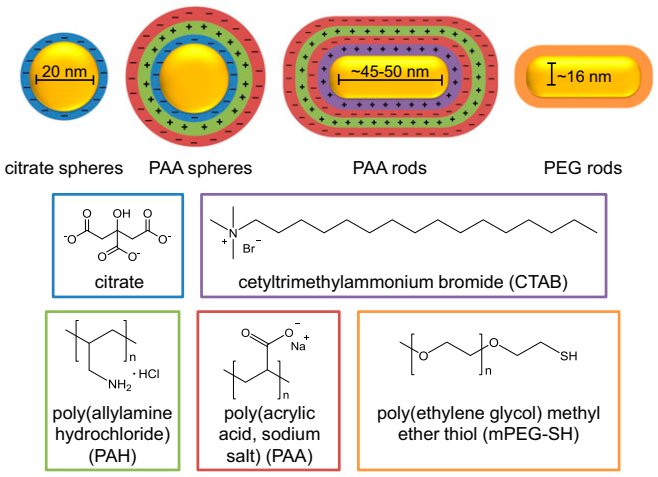

Fig. 1. Schematic of gold nanoparticles and their coatings. Cartoon depictions of the gold nanoparticle types used. Colors surrounding the nanoparticles correspond to surface coating layers of the color-coded molecules at the bottom of the figure. The surface charge of each layer is indicated by minus and plus signs within the layers.

from 0.05 to 10 nM, and cell type and NP shapes vary widely as well (23–26).

Despite the ever-accumulating data regarding the effects of NPs and their physical parameters on cells, more rigorous studies that include long-term exposure at low, realistic dosages of different shapes and surface chemistries of Au NPs are necessary (26–28). Herein, we report the in vitro long-term (20 wk) changes in human cell lines exposed to well-characterized Au NPs with varying shapes and surface coatings under both chronic (exposure to Au NPs continuously over 20 wk) and nonchronic (initial acute cell exposure to Au NPs, followed by 20 wk in NP-free cell media) conditions. Cell viability, proliferation, morphology and the expression of an array of genes were examined, along with the levels of cellular uptake of each NP type. We studied the effects at the molecular and cellular levels for long-term, low doses of Au NPs (0.1 nM) applied to a commonly used human skin cell model (human dermal fibroblasts) to mimic unintentional environmental exposure. The ability of cells to recover properly from acute NP exposure over time is tested by the nonchronic experiments.

Results

Nanoparticle Characterization and Cell Exposure. To determine the long-term effect of both surface coating and shape of Au NPs on human dermal fibroblasts (HDFs), two shapes of Au NPs (nanospheres and nanorods), each with two different surface coating types, were synthesized and characterized. A schematic depicting the different types of Au NPs used in this study is shown in Fig. 1. Nanospheres (18 nm) were made with either citrate or poly(acrylic acid) (PAA) coatings (denoted as citrate spheres and PAA spheres) and nanorods (average 48 × 16 nm) were made with either PAA or PEG coatings (PAA rods and PEG rods). Citrate spheres are simply as-made Au NPs, and anionic PAA was chosen as the second sphere coating to keep surface charge constant. Gold nanorods have cetyltrimethylammonium bromide (CTAB) bilayers on their surfaces after synthesis and are typically made biocompatible by either overcoating it with polyelectrolytes or by displacing the CTAB bilayer. To compare two NPs with nearly identical surfaces but different shapes, nanorods triple-coated over the CTAB layer to a final layer of PAA (PAA rods) were made to compare with PAA spheres. A well-studied surface functionalization on nanorods is PEG, which, when conjugated to a thiol, covalently binds to the gold surface and can displace CTAB; this neutral, antifouling coating was the choice for the final NP type tested. After synthesis of the Au NP cores, the NPs were fully characterized by UV-visible (UV-Vis) spectroscopy (*SI Appendix*, Fig. S1), dynamic light scattering (DLS)/zeta potential measurements (*SI Appendix*, Tables S1 and S2) and transmission electron microscopy (TEM) (*SI Appendix*, Fig. S2). To verify that the subsequent surface modifications were successful, a combination of UV-Vis, DLS and zeta potentials were used after each step. These NPs were then used for chronic and nonchronic exposure experiments; a schematic depicting the week-long cycle for exposing HDF cells to NPs over the course of 20 wk (5 mo) is shown in Fig. 2.

Gold Nanoparticles Are Generally Not Cytotoxic. Viability and proliferation analyses were conducted on HDF cells exposed to 0.1 nM of NPs for 3, 7, and 14 d and on samples exposed under nonchronic conditions as well, after receiving NPs for the first 24 h only. Very little difference was observed in cell viability between NP-treated and control samples in both chronic and nonchronic exposures (*SI Appendix*, Fig. S3). Cells treated by PAA rods and PAA spheres under chronic conditions were statistically different from controls after 7 (rods only) and 14 d (both), but the viability was above 92% in all instances. Cells were counted at each passage throughout the long-term study, and cell numbers stayed steady for each sample type. Cell proliferation rates at 3, 7, and 14 d did not significantly vary from control for any NP sample type, indicating no effect of NPs on proliferation. No statistical difference was measured between chronic and nonchronic samples of the same NP type.

Relative NP Uptake Levels Are Governed by Surface Coating and Shape. The number of NPs ingested by cells was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES) for chronic incubations of each NP type (Fig. 3*A*). Additionally, the data are shown in *SI Appendix*, Fig. S4, in terms of surface area and volume of the NPs per cell (assuming that the ends of the nanorods are spherical). Because the spheres and nanorods are similar in dimensions, the same trend is observed regardless of metric (number of NPs, surface area, or volume). Nonchronic samples were not run because the low levels of NPs were undetectable by ICP-OES. Uptake measurements were compared between short-term (72 h) and long-term (chronic) exposures by measuring the gold content inside cells (*SI Appendix*, Table S3). At both time points, PAA spheres were

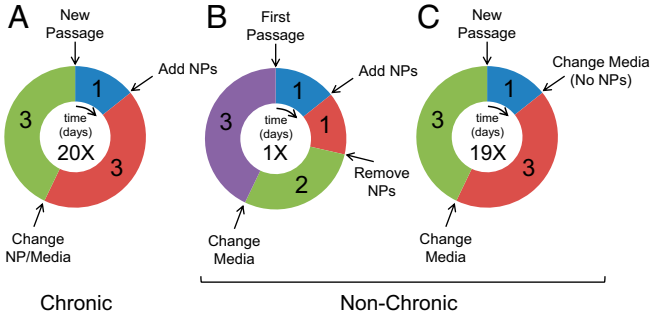

Fig. 2. Schematics showing the cell exposure procedure for chronic and nonchronic samples. (*A*) Chronic cell samples were plated, and NPs/media (0.1 nM NPs) were added after 24 h. NP/media solutions were changed 3 d later, and cells were passaged after an additional 3 d to start a new cycle. (*B*) Nonchronic cell samples were plated and NPs/media (0.1 nM NPs) were added after 24 h. NP/media was changed to just media after another 24 h. Media was refreshed 2 d later, and another 3 d later, the cells were passaged. (*C*) After the first week, the nonchronic subsequent passage cycles followed the chronic sample schedule, with no NPs added. Controls followed same schedule, with no NPs ever added.

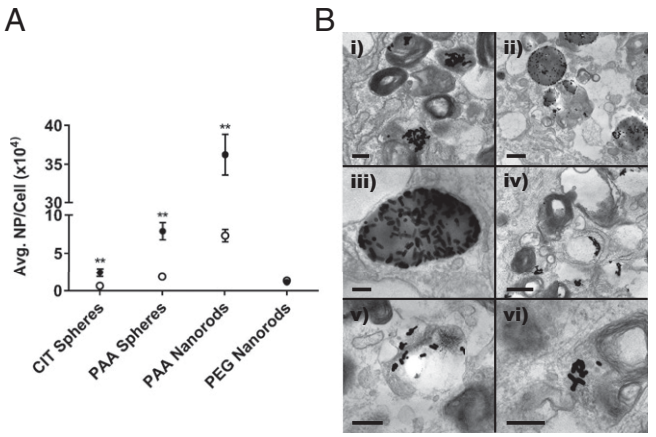

Fig. 3. Nanoparticle internalization by cells. (*A*) Average number of NPs per cell measured by ICP-OES analysis for gold content at 72 h (○) and 20 wk (●). Stars indicate significant difference between short- and long-term samples of the same NP type. $*P < 0.05$, $**P < 0.01$. Error bars represent one SD from the mean of three measurements. (*B*) Transmission electron micrographs of HDF cells under chronic NPs exposure. Samples exposed to the following: (*i*) Citrate spheres. (Scale bar: 200 nm.) (*ii*) PAA spheres. (Scale bar: 500 nm.) (*iii*) PAA rods. (Scale bar: 100 nm.) (*iv–vi*) PEG rods after 20 wk. (Scale bars: 500, 200, and 200 nm, respectively.)

endocytosed at higher levels than citrate spheres, and PAA rods were ingested more than PEG rods. At 72 h, citrate spheres were the least preferred for uptake, and at 20 wk, PEG rods were the least preferred. Overall, PAA rods were taken up much more than all other NP types (7.3×10^4 NP per cell at 72 h and 3.6×10^5 NP per cell at 20 wk). The general trend (PAA rods > PAA spheres > citrate spheres ~ PEG rods) is consistent for each time point.

Nanoparticles Exert Some Changes in Morphology of HDF Cells. Cell imaging was done to compare the short-term (after 24 and 48 h) and long-term effects (after 20 wk) of NP exposure on cell morphology using confocal fluorescence microscopy for both chronic and nonchronic exposures. Typical micrographs for each time point are shown in *SI Appendix*, Figs. S5–S7. Average cell area was calculated for each sample type (*SI Appendix*, Fig. S8). At 24 h, the cells exposed to PEG rods were smaller than control cells, and at 48 h, all nonchronic samples had significantly decreased cell areas. The cell areas were the same as controls in the long-term samples, except for nonchronic PAA rods and both nonchronic and chronic PEG rods exposures, which had increased cell area relative to controls.

PEG Rods Do Not Accumulate in Vesicles but Instead Escape to the Cytosol. Examination of fixed cell samples by TEM found no NPs still present in the cells 20 wk after acute exposure (nonchronic). However, NPs were found in all of the chronically exposed samples, with their spherical or rod shapes conserved. It was observed that though many NPs could be found together in large endosomes/lysosomes in cells treated with citrate spheres, PAA spheres, and PAA rods, most of the PEG rods were located directly inside of the cytoplasm or in vacuoles (Fig. 3*B*). Dark lamellar bodies were present in all sample types (both chronic- and nonchronic-exposed cells), but were also very prevalent in the control cells. Therefore, their presence cannot be linked to NP exposure.

More Changes in Gene Expression Are Measured in Nonchronic Samples than in Chronic Samples. A total of 84 genes related to stress and toxicity pathways were selected for gene expression level measurement in HDF cells after chronic and nonchronic exposures. No sample amplification or nonspecific amplification was observed in 12 sets of primers, and the corresponding genes were not considered (*AQP2*, *AQP4*, *CD40LG*, *FTR*, *CRP*, *EPO*, *IFNG*, *IL1A*, *IL1B*, *IL8*, *MMP9*, and *TNF*).

Twenty-five from 72 genes (~35%) were significantly differentially expressed in at least one sample type (chronic or nonchronic, any NP type). For the chronic exposure, 12 genes presented altered levels of expression compared with control cells vs. 19 genes in the nonchronic condition. The nonchronic PEG rods exposure induced the most gene expression changes (18 in total). A total of six genes expressed differently were common between both types of exposure. Functional network analysis of the genes modulated by nonchronic PEG rods exposure are shown in Fig. 4. The specific genes and related functional pathways with accompanying fold changes/*P* values are tabulated in *SI Appendix*, Tables S4 and S5. The comparisons between sample conditions (chronic and nonchronic exposures) for the same gene differentially expressed for at least one sample type is represented in Table 1.

Discussion

The goal of this study was to supply new insights about how engineered NPs with different shapes and surface coatings affected cells at different levels over a long period at a low dose.

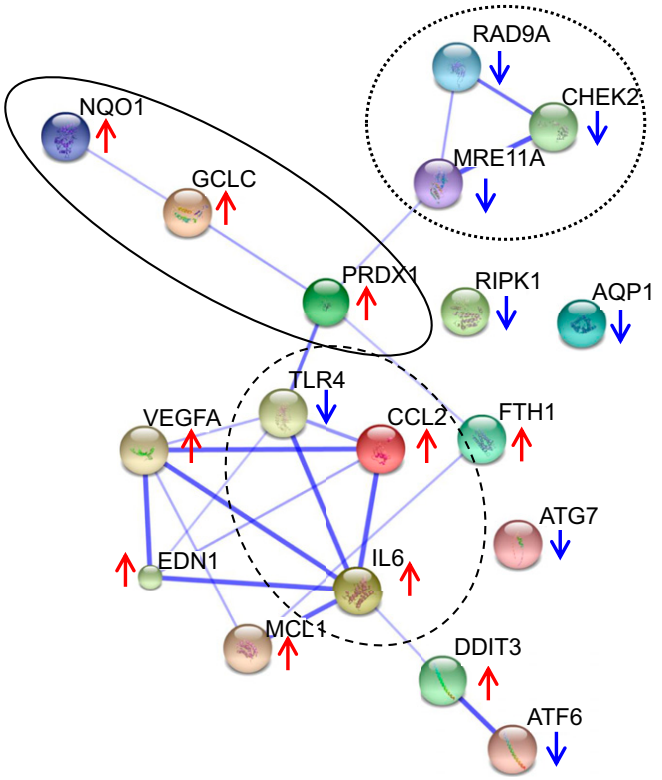

Fig. 4. Network of known functional interactions between the genes significantly differentially expressed in nonchronic PEG rod samples built using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database version 10 at medium confidence levels (scores between 0.4 and 0.7). The relative thickness of lines connecting proteins indicates the confidence score of their interaction. Red arrows pointed up indicate genes that are up-regulated, and blue arrows pointed down indicate genes that are down-regulated. The solid line encircles a set of genes that are all related to oxidative stress; the dotted line encircles a set of genes all related to cell cycle checkpoint/arrest; and the dashed line encircles a set of genes all related to inflammatory response. Protein nodes, which are enlarged, indicate the availability of 3D protein structure information.

Cell viability and proliferation tests over 2 wk confirmed that a 0.1-nM concentration of Au NPs, independent of NP type, were indeed generally noncytotoxic; cell viability never dropped below 92% for any sample type. The slight reduction in the cell number can be related to the increase of stress levels triggered by NPs instead of to the cell death process. Indeed, the most remarkable decrease on viability was observed in cells exposed to PAA rods (after 7 and 14 d) in a chronic condition in which the up-regulation of genes related to antioxidant pathways was not observed. Furthermore, a stress-related gene (*HSPA5*) was overexpressed by such NPs. The concentration is a major factor in ensuring that the NPs do not induce toxicity, as another group has found similarly sized (13 nm) citrate Au NPs to induce apoptosis at ∼0.1 mg/mL (7 nM) (9). However, earlier work in our laboratory with the same HDF cells has found 20 nm citrate Au NPs to induce negligible changes to viability from 0.1 to 2.0 nM (12, 23).

At the cellular level, we observed small but statistically significant changes in cell morphology between controls and NP-exposed cells at each time point tested. Actin structures did not appear to change in any discernible way. However, at 24 h, cell area decreased significantly for PEG rod-exposed cells and all nonchronic NP-exposed samples decreased in cell area from control at 48 h. After long-term culture, the decrease in cell area recovered in the nonchronic samples and cell area actually increased for cells exposed to PAA rods in a nonchronic condition and for both chronic and nonchronic PEG rods compared with controls. The large cell area in these samples after 20 wk correlates with high levels of PAA rods in the cells, but PEG rods were found in the lowest numbers. The large cell area may correspond to relatively large gene expression changes induced by PEG rods. Interestingly, others have found much higher levels of PEGylated Au spheres to induce no morphological changes to human umbilical vein endothelial cells, even at levels that induce oxidative stress in short-term studies (29). Pernodet et al. (8) and Mironava et al. (9) report a decrease in cell area with increasing concentrations of 13 nm citrate Au NPs and an increase in cell aspect ratio with 13 and 45 nm citrate Au NPs; again, these are at much higher concentrations. Yang et al. (12) also found that 20 nm citrate Au NPs disrupted F-actin fibers in HDF cells, but only for 1.0-nM particle concentrations, 10× more compared with the present work. Increases in the cell size over time were observed, but were similar in all of the samples, including the control. As the HDF cells age, they tend to enlarge, and at the end of the study the cells were nearing senescence (30). However, the cells were still dividing and growing as the number of cells in each passage stayed steady. Additionally, the *CDKN1A* gene (an indicator of senescence when up-regulated) was not differentially expressed by any NP exposed samples compared with controls.

Uptake of the NPs by HDF cells was highly dependent on both surface coating and shape. PAA spheres were taken up by cells ∼3× more than citrate spheres at 72 h and 20 wk, and PAA rods were taken up ∼6× and 30× more than PEG rods at both time points, respectively. PAA-coated spheres were also found to be more readily endocytosed by SK-BR-3 cells than citrate spheres, and PEG coating has been shown to decrease the uptake of NPs (31, 32). Interestingly, nanorods were highly preferred for uptake by HDF cells over spheres with PAA coatings. From 4 to 4.5× more PAA rods were taken up than PAA spheres at both time points. This result contradicts the general consensus that nanorods are more difficult for cells to endocytose than spheres of similar sizes, but there are few studies in which shape effects are measured with surface chemistry being properly controlled (26–28, 33, 34). By comparing cellular uptake levels of spheres and

Table 1. Up- and down-regulated genes in both nonchronic and chronic exposures

Gene symbol	Functional pathway	Nonchronic				Chronic			
		Citrate	PAA spheres	PAA rods	PEG rods	Citrate	PAA spheres	PAA rods	PEG rods
IL-6	Inflammatory response								
VEGFA	Hypoxia								
CCL2	Inflammatory response								
PRDX1	Oxidative stress								
FTH1	Oxidative stress								
MCL1	Cell death (apoptosis)								
GCLC	Oxidative stress								
EDN1	Osmotic stress								
DDIT3	Unfolded protein response/DNA damage signaling								
NQO1	Oxidative stress								
SLC2A1	Hypoxia								
NFAT5	Osmotic stress								
GADD45G	DNA damage signaling								
TNFRSF10A	Cell death (necrosis/apoptosis)								
TP53	DNA damage signaling								
BBC3	Unfolded protein response								
RAD9A	DNA damage signaling								
ATG7	Cell death (autophagy)								
CHEK2	DNA damage signaling								
ATF6	Unfolded protein response								
MRE11A	DNA damage signaling								
RIPK1	Cell death (necrosis)								
TLR4	Inflammatory response								
HSPA5	Unfolded protein response								
AQP1	Osmotic stress								

Up-regulated genes are in red, and down-regulated genes are in blue. Genes included have an average FC ≥ 1.5 or ≤ −1.5 and $P < 0.05$ in at least one sample type in the chronic and/or nonchronic exposures.

rods both with PAA coatings, the role of a single variable (shape) is clearer.

The NP uptake levels in cells exposed to chronic conditions were slightly increased after 20 wk compared with 72 h. The balance between factors such as passaging the cells, cell division, and exocytosis kept the NPs inside cells more or less steady between time points. No NPs were observed in nonchronic samples after 20 wk. On the contrary, many NPs were found in cells in chronic samples. It is well known that NPs in this size range are uptaken by cells via receptor-mediated endocytosis and usually stay inside endosomes and lysosomes (35). However, PEG rods were almost exclusively found outside of distinct vesicles, unlike the other NP types tested. Although there are limitations to analysis of uptake via TEM (slice-by-slice imaging), these qualitative observations have been observed before and quantified in other cases by more sophisticated analyses (36). Occasional lysosomal escape has been reported with positively and negatively charged gold nanorods before, and it was found that PEGylated gold nanospheres escaped into the cytoplasm of macrophages and lung cells (PEG NPs escaped more than citrate NPs in the case of the lung cell study) (36–38).

At the molecular level, the very low concentration of NPs used here (0.1 nM) was able to impact the gene expression of HDF cells. A comparison between exposure conditions showed a larger number of changes occurred after the nonchronic condition than after its chronic counterpart. This result suggests that the time of exposure had a large impact on gene regulation after 20 wk. Six genes presented altered expression levels independent of the exposure type; two of them, *VEGFA* and *CCL2* (related to cell metabolism/angiogenesis and inflammation, respectively), showed different expression trends, being up-regulated after the nonchronic exposure and down-regulated in the chronic one. Genes related to antioxidant pathways (*PRDX1* and *NQO1*) and osmotic stress (*EDN1*), as well as the *HSPA5* gene, were overexpressed in both chronic and nonchronic conditions. This ER-resident chaperone is a member of the heat shock protein 70 (HSP70) family, and is implicated as a key molecule in the protein folding and assembly process within the ER. *HSPA5* overexpression is induced by stress conditions to promote cell survival. High levels of this protein are observed in a huge variety of tumors and are related to drug resistance promotion in cancer treatment (39).

For chronic Au NP exposure, different expression profiles were observed according to NP type. Cells treated with citrate spheres did not show significant changes in the regulation of any gene evaluated. PAA rods exposure down-regulated genes related to inflammation (*CCL2*), metabolism (*SCL2A1* and *VEGFA*), and cell cycle regulation and apoptosis (*GADD45A*, *TNFRSF10A*, and *TP53*) and led to the up-regulation of *HSPA5*. PEG rods also down-regulated genes related to apoptosis (*TNFRSF10A* and *BBC3*). However, genes related to antioxidant pathways such as *NQO1* and *PRDX1* were up-regulated by PEG rods exposure but not by PAA rods.

Genetic changes were still observed in HDF cells, even 20 wk after any NP exposure, in the nonchronic data. A pattern of gene expression among the different NP types can be described. Overall, genes related to antioxidant, proteotoxic stress, and antiapoptotic pathways were up-regulated. However, genes related to the maintenance of cell homeostasis were down-regulated. Alterations in these genes are also reported in a variety of human cancers and other pathologies (39–41). As in the chronic exposure, the augmented oxidative stress triggered by PAA rods in the nonchronic exposure was not accompanied by the augmentation in the levels of antioxidant defense genes presented in our array.

Though PEG rods were the least endocytosed NPs after long-term culture, the nonchronic PEG rods exposure induced the most gene expression changes. IL-6 was ∼12× more expressed than in control cells. In vitro and in vivo studies have also reported the ability of PEG-coated Au NPs (spheres) to induce some inflammatory mediators such as IL-6 (42, 43). High levels of this cytokine have been related to age-related diseases, such as cancer. Although some different genes were down-regulated compared with other NP types in nonchronic exposure samples, gene categories affected by PEG rods were similar (oxidative stress, inflammation, cell cycle checkpoint/arrest, and cell death). As shown by the functional network analysis, genes grouped by pathway are mostly up- or down-regulated similarly (Fig. 4). PEG is a polymer often used to increase circulation time in vivo and is considered to be highly biocompatible (44). However, we have found that PEG rods are able to interact more freely with the contents of the cytoplasm without a barrier (endosomes or lysosomes). It has been reported that the exocytosis process of NPs translocated into the cytoplasm is much more difficult than for NPs inside the endosomes or lysosomes (45); this is most likely the reason behind the enhanced gene expression changes relative to the other NP types.

Shape did not have as large of an effect on gene expression as did surface coating. Even though the PAA rods were taken up by cells at levels many times higher than their spherical counterparts, they produced similar amounts of expression changes. However, in nonchronic samples, there are similar patterns of expression between citrate spheres and PAA spheres. Half of the genes with altered expression in the citrate spheres-treated samples were also altered by PAA spheres and PAA rods, but two of the genes (*PRDX1* and *FTH1*, related to oxidative stress) are not significantly altered by PAA rods.

Our findings show that both chronic and nonchronic Au NPs exposures were able to disrupt cell homeostasis via altered levels of gene expression related to cell cycle regulation and oxidative stress after long-term. The exception was citrate spheres, because this type of NP did not have a significant influence on the levels of gene expression over 72 genes analyzed. However, this is true considering just the chronic exposure. Similar gene expression changes are often observed during the onset and progression of cancer (46). Hypoxia, oxidative stress, and inflammation in fibroblasts are especially of concern because these cells are known have a large role in the homeostasis of surrounding cells in the connective tissue (47).

The cell response to insults or stressors (in this case, the NPs) is an attempt by the cell to ensure survival. Chronic, low doses may lead to a prolonged change in cell physiology as part of an adaptive response to the long-term effects of a specific stimulus (48). The results obtained from the chronic exposure in the present study show that cell changes on the molecular level to reached an adapted state (new homeostasis) to continual stress. In contrast, the chronic exposure (6 mo) to low doses of industrial MWCNTs (0.5 μg/mL) did not show any adaptive mechanism induction in cells after long-term treatment (49). The nonchronic Au NPs exposure generated a quite different gene expression profile compared with chronic. The results obtained are intriguing because the larger and sustained stress responses can be detected even after 20 wk without NP stimulation. The degree of stress activation was not related to the longest time of exposure.

This study demonstrated the long-term effects of acute and chronic Au NPs exposures at low dose on human cells. Although no significant cytotoxicity was observed, Au NPs of varying shapes and surface coatings have an impact on cell morphology and on gene regulation. The cellular responses are quite different when the two types of exposure are compared. The initial decrease in cell area was recovered in nonchronic samples over time, which indicates that some processes that are affected by the exposure to NPs for short periods of time can be recovered in the long run. However, the long-term stress response, especially that induced by nonchronic exposure, is a concern and needs to be further explored.

We demonstrated that the NP surface chemistry was a determinant factor in driving gene expression changes. By analyzing 72 genes related to stress and toxicity pathways, citrate spheres seems to be relatively benign to HDF cells, especially

chronically at very low doses. PAA rods induce oxidative stress without concomitant antioxidant defense activation and may cause future cell damage. PEG-coated rods by far induced the largest modifications to gene expression. These NPs are able to travel into the cytoplasm of the cells, which is possibly the root cause of substantial stress and inflammation induction observed after long time exposure. This study, relative to others, has also shown that the effect of NP shape on uptake levels may be highly cell type- and surface moiety-dependent. Depending on surface chemistry, Au NPs at low doses that appear benign by various measures may still destabilize the regulatory responses of cells to induce cellular stress long after NP removal.

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