Citrate-stabilized gold nanoparticles as negative controls for measurements of neurite outgrowth

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ARTICLE INFO

Article info
Received 24 June 2014
Accepted 8 October 2014
Available online 23 October 2014

Keywords:
Assay controls
Gold nanoparticles
Metabolic activity
Nanoparticle stability
Negative controls
Neurite outgrowth

ABSTRACT

Gold nanoparticles (AuNPs) are promising candidates for medical diagnostics and therapeutics, due to their chemical stability, optical properties, and ease of functionalization. Citrate-stabilized reference materials also have potential as negative controls in toxicology studies of other nanoparticles. Here we examine the impact of 30 nm particles on the in vitro development of rat-cortex neural progenitor cells (NPCs), which mimic aspects of the developing neurological environment. AuNPs dispersed in a low-serum culture medium initially agglomerated, but then remained stable during a three day incubation period, and agglomerated only slightly during a ten day incubation period, as determined by dynamic light scattering. Transmission electron microscopy indicated the presence of individual nanoparticles at all timepoints examined. Fixed cells were cross-sectioned by ion milling and imaged by scanning electron microscopy and helium-ion microscopy to evaluate particle incorporation. Individual nanoparticles could be resolved inside cross-sectioned cells. AuNPs were incubated with developing NPCs for ten days at concentrations of 0.5 μg/mL Au, 0.1 μg/mL Au, or 0.05 μg/mL Au. Adenosine triphosphate levels, as determined by bioluminescence measurements sensitive to low cell numbers, were not affected by AuNPs and the particles did not interfere with the assay. Multiple endpoints of neurite outgrowth were not altered by AuNPs, in particular, total neurite outgrowth per cell, a sensitive measure of neuronal development. Slide-level comparisons demonstrated the consistent response of NPCs to gold nanoparticles and a positive control chemical, neuroactive lithium. These results indicate that 30 nm citrate-stabilized AuNPs could serve as negative-control reference materials for in vitro measurements of neurite outgrowth.

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1. Introduction

One persistent challenge in the interpretation of in vitro nanoparticle toxicity studies is that novel materials are examined for their effect on cellular endpoints without positive or negative assay controls. Chemical controls provide confirmation that the cells are behaving normally, for example, by responding to a known neuroactive chemical. Nanoparticle controls might provide additional information by allowing the effects of material or surface modification to be separated from physical particle dimensions. Nanoparticle control materials must be physically and chemically characterized, sterile, and dispersible in the required culture medium. Ideally, their uptake by cells should be readily demonstrable. Finally, their toxicological effects on specific in vitro cell models and endpoints should be reproducible.

Gold nanoparticles (AuNPs) might be employed within the central nervous system for imaging (Hutter and Maysinger, 2011), delivery (Prades et al., 2012), tracking implanted stem cells (Wang et al., 2006; Ricles et al., 2011) and inhibiting amyloid-beta fibrillization (Liao et al., 2012). Diagnostic and therapeutic applications are expected to utilize particles larger than 5 nm, which are generally expected to be nonreactive (Alkilany and Murphy, 2010). Citrate-stabilized AuNPs, such as the reference materials developed by the National Institute of Standards and Technology (NIST), also have potential as control materials. These materials...
were previously demonstrated not to induce oxidative nucleic acid damage, suggesting their application as negative controls for genotoxicity studies (Nelson et al., 2013). Here, we examine them in the context of developmental neurotoxicity studies.

Neural cells have numerous complex functions, only some of which have been explored by in vitro nanotoxicology studies. Exposure to 25 nm poly(ethylene glycol)-modified or citrate-stabilized AuNPs for 24 h was demonstrated to decrease neurotransmitter release in murine chromaffin cells (Love and Haynes, 2010; Love et al., 2012). Spherical 23 nm AuNPs were found to activate microglial cells after 24 h exposure (Hutter et al., 2010). When applied at 800 particles per cell (0.065 μg/mL Au), 20 nm AuNPs increased the fraction of proliferative cells in neural progenitor cell cultures (Soderstjerna et al., 2013). Exposure to 4 nm AuNPs coated with poly(methacrylic acid) decreased neurite outgrowth in rat pheochromocytoma cells (Soenen et al., 2012) and clusters of nanoparticles were hypothesized to sterically hinder the cytoskeleton. Another potential mechanism results from the ability of metal nanoparticles to enhance cellular formation of reactive oxygen species (Nel et al., 2006). Oxidative stress has been reported to disrupt cytoskeletal proteins in the central nervous system (Allani et al., 2004) and inhibit neurite outgrowth (Neely et al., 1999). Microarray analysis of rat hippocampal tissue following inhalation of 7 nm AuNPs recently indicated downregulation of genes related to the cytoskeleton and neurite outgrowth (Balasubramanian et al., 2013). However, larger AuNPs relevant to biomedical applications have not been investigated for disruption of neurite outgrowth in a complex in vitro cell model.

In vitro developmental neurotoxicity studies involve assaying key processes (e.g., proliferation, migration, differentiation, neurite outgrowth, or synaptogenesis) (Croffon et al., 2011) that are highly conserved among species (Lein et al., 2005). Neurite outgrowth is a defining feature of neural morphology and is critically linked to neural connectivity (Radio and Mundy, 2008; Robinette et al., 2011). Here, we employ neurons which develop from progenitor cells isolated from the rat cortex. Progenitor cells are an emerging model that mimics many aspects of the developing neurological environment (Moors et al., 2009; Breier et al., 2010). In particular, astrocytes can inhibit (McKeon et al., 1991) or promote (Oh et al., 2009) neurite outgrowth, suggesting their importance to neurite outgrowth assays. For the cells employed here, both the fraction of neurons and the fraction of astrocytes increased significantly from three days in culture to ten days in culture (by 2 × for neurons, by 20 × for astrocytes), whereas the fraction of progenitor cells decreased over the same time period (Jeerage et al., 2012). After ten days in culture, two measures of neurite outgrowth were sensitive to the neuroactive chemical lithium at concentrations that encompass the therapeutic range of lithium (Jeerage et al., 2012). Importantly, these results were observed at non-cytotoxic exposures, where metabolic activity was similar to control cultures.

In this work, we examined the effect of 30 nm citrate-stabilized AuNPs on neurite outgrowth and their potential to serve as negative controls for in vitro toxicity studies. Such particles are within the size range relevant to biomedical applications. For example, 20 nm particles maximize gene delivery to cells (Elbakry et al., 2012), while 40 nm particles are described as favorable for imaging, due to their absorption cross section (Jain et al., 2006). We established cellular uptake by serially sectioning individual cells with a focused ion beam. Individual particles were imaged within the cellular cross sections with high resolution by helium-ion microscopy. Adenosine triphosphate levels and multiple measurements of neurite outgrowth indicated that biomedically-relevant particle concentrations had no detrimental effects on these parameters after ten days. Cross-slide comparisons of cultures exposed to AuNPs (0.5 μg/mL Au) indicated the reproducibility of total neurite outgrowth measurements, with a coefficient of variation below 10%, similar to cultures exposed to the previously-established chemical control, lithium (3 mmol/L Li⁺). These results suggest that citrate-stabilized AuNPs are promising negative controls for neurite-outgrowth-based developmental neurotoxicity measurements.

2. Materials and methods

2.1. Neural cell culture

Rat cortex neural stem/progenitor cells (NPCs) from Stem Cell Technologies were cultured in proliferation medium (NeuroCult NS-A proliferation medium supplemented with recombinant human epidermal growth factor, recombinant human-fibroblast growth factor, and heparin) or differentiation medium (NeuroCult NS-A differentiation medium supplemented with 50 units/mL penicillin and 50 μg/mL streptomycin). Thawed neurospheres were resuspended in proliferation medium and passed into a single-cell suspension after 24 h. Proliferation medium was partially exchanged after 48 h and cells were seeded in differentiation medium after an additional 48 h at a density of 1.25 × 10⁵ cells/cm². Culture surfaces were treated with 15 μg/mL poly-L-ornithine to promote cell attachment.

2.2. Nanoparticle exposure

NPCs were allowed to attach to the culture surface for 4 h prior to nanoparticle exposure. For cells cultured in 8-well chamber slides (0.8 cm², Lab-Tek), 100 μL of each stock suspension was added to wells containing 300 μL of differentiation medium to give final exposure concentrations of 3 mmol/L Li⁺, 0.05 μg/mL Au, 0.1 μg/mL Au, and 0.5 μg/mL Au. For cells cultured in opaque white 96-well assay plates (0.32 cm², Corning), 25 μL of each stock suspension was added to wells containing 75 μL of differentiation medium to give the same final exposure concentrations. Culture medium was not exchanged during the ten day culture period, so the nanoparticle concentration remained constant. Samples obtained from cell cultures after one, three, seven, and ten days indicated the continued presence of suspended gold nanoparticles by dynamic light scattering (see next section for experimental details).

Citrate-stabilized, zero-valent, spherical gold nanoparticle reference materials have nominal dimensions of 10 nm (not used here), 30 nm (https://www-s.nist.gov/srmors/reports/8012.pdf), and 60 nm (https://www-s.nist.gov/srmors/reports/8013.pdf) and are available to other researchers through NIST (RM8011, RM8012, RM8013). Nanoparticle stock suspensions were prepared by adding 200 μL of reference material (50 μg/mL) to a centrifuge tube containing 1.8 mL of differentiation medium while vortexing at 1900 rpm. Vortexing continued for 30 s after adding the nanoparticles. This protocol was developed to disperse citrate-stabilized gold nanoparticles at their primary particle size by quickly surrounding the particles with bovine serum albumin (Zook et al., 2011). This stock suspension (5 μg/mL) was diluted as needed.

2.3. Dynamic light scattering (DLS)

Dulbecco’s Modified Eagle Medium (DMEM) without phenol red was supplemented with Neurocult NS-A differentiation supplements to create a medium that contained the same serum components as NS-A differentiation medium but without appreciable...
absorption. Particles were dispersed (5 μg/mL Au) as described earlier and 45 μL aliquots were added to a low-volume quartz cuvette (ZEN2112) for size measurements with a Zetasizer Nano (Malvern). This instrument employs a helium–neon laser (633 nm) and measures the scattered light at 173° (backscatter). Measurements were performed at 20 °C. Particle suspensions were added to a culture dish and incubated (37 °C, 5% CO₂) between measurements to replicate cell culture conditions. Prior to each subsequent measurement, the suspensions were slowly rotated on an orbital shaker (50 rpm) for 30 min to bring them to room temperature and to ensure that large agglomerates, which might have settled to the bottom, would be sampled.

2.4. Transmission electron microscopy (TEM)

Particles were dispersed (5 μg/mL Au) as described earlier. Immediately after preparation, 15 μL aliquots were drop cast onto an ultrathin (less than 3 nm) carbon membrane on a holey carbon coated copper support grid. Grids prepared with fresh particle suspensions were simply dried overnight and were challenging to image due to the presence of organic materials and thick salt crystals, which obscured the particles and caused charging of the sample. Particle suspensions were added to a culture dish and incubated (37 °C, 5% CO₂), and additional grids were prepared after nine days. To minimize the formation of thick obscuring salt crystals in these samples, particles were allowed 30 min to settle after drop casting, then the remaining liquid was wicked from the surface before allowing the grids to dry overnight. These samples still contained salt crystals, but the majority of the grid could be imaged.

2.5. Scanning electron microscopy (SEM) and focused ion beam (FIB) milling

NPCs were grown on cover slips and exposed to particles (5 μg/mL Au) for 10 days. Cells were fixed by incubation with 4% paraformaldehyde for 60 min (21 °C), then dehydrated and air-dried. The dehydration series consisted of 25% ethanol for 120 min (2 solution changes), 50% ethanol for 60 min, 80% ethanol for 30 min (3 solution changes), and 100% ethanol for 30 min (3 solution changes). The remainder of each solution was phosphate-buffered saline.

After dehydration, NPCs were coated with approximately 25 nm of platinum using argon-ion sputtering to minimize charge accumulation during FIB milling. The film was sputtered for 7 min from a platinum target at a rate of approximately 0.06 nm/s with a Penning gun at an acceleration voltage of 10 kV. The plated cells and glass slide were then cross-sectioned by use of a focused gallium beam. The acceleration voltage was 30 kV and the gallium beam current was approximately 240 pA. The gallium beam was normal to the sample surface and the rate of milling for the cells and glass was approximately 20 μm/min, at a nominal dose of 4 nC/μm² and dwell time of 6 ms. SEM images were taken at approximately 200 nm-depth intervals with the gallium beam deactivated. These images were acquired at 54° to the milling direction. Fiducial marks were gallium beam milled with similar parameters to locate the cross-sectioned cells in subsequent imaging.

2.6. Helium ion microscopy (HIM)

Cellular cross sections were imaged at 45°. The acceleration voltage of the helium ions was 35 kV and the nominal beam current was 0.3 pA. Secondary electrons produced from the sample were collected with an Everhart-Thornely detector, with a point dwell period of 10 μs.

2.7. Adenosine triphosphate (ATP) evaluation

In the CellTiter-Glo Luminescent Cell Viability Assay (Promega), the enzymatic conversion of beetle luciferin to oxyluciferin is determined. Luminescence intensity was measured with a Synergy HT Multi-mode Plate Reader (Biotek), background-corrected, and normalized to controls. Results are based on twelve wells from two independent cultures. Since all light was collected by the luminometer, nanoparticle interference was evaluated by measuring a standard curve without and with a freshly mixed nanoparticle suspension (final concentration 0.5 μg/mL Au). For the standard curves, ATP concentrations were 0.075 μmol/L, 0.75 μmol/L, 1.5 μmol/L, and 2.25 μmol/L.

2.8. Immunocytochemistry and image acquisition

Cells were fixed by incubation with 4% paraformaldehyde for 30 min (21 °C), then incubated with 0.3% Triton-X 100 for 30 min (21 °C) to permeabilize the membrane. Fixed cells were first incubated with mouse monoclonal anti-β-III-tubulin (1:1000; Clone TuJ-1, FitzGerald) and rabbit monoclonal anti-GAP (1:1000; Clone EP672Y, Abcam) in 10% normal goat serum for 2 h (37 °C). Cells were next incubated with goat anti-mouse AlexaFluor 488 IgG (1:100; Invitrogen) and goat anti-rabbit AlexaFluor 594 IgG (1:100; Invitrogen) in 2% normal goat serum for 30 min (37 °C). Cells were finally incubated for 60 s with Hoechst 33342 (5 μg/mL). Immunostained cells were imaged with a 20× objective on a Nikon TE-2000S inverted microscope equipped with an EXFO X-cite 120 metal-halide arc lamp and standard excitation and emission filters (Omega Optical). All images were captured by a 12-bit monochrome camera (Photometrics ES2) with Metamorph Software (Molecular Devices). Unbiased image regions were chosen by advancing the stage until a non-overlapping region was visible; regions were rejected if fewer than 5 neurons or more than 20 neurons were visible.

2.9. Neurite outgrowth evaluation

Neurites were traced on an average basis with Neurolucida (MBF Biosciences) and a full description of the approach, including traced image samples, has been published (Jeerage et al., 2012). In this approach, the cell soma, which is strongly stained, and all neurites within an image are traced, even if the cell soma is not visible in the image. Consistent intensity scaling, seed placement, and gap tolerance settings were employed within the software. For each traced image, neurons were identified by observation as β-III-tubulin positive neurites extending from a cell body (Hoechst 33342 positive) that was bounded by β-III-tubulin. Neurite outgrowth was summed for all images and divided by the total number of identified neurons to determine the total neurite outgrowth per cell for each well. For each well, 10 images were analyzed, with an average of 105 neurons (minimum of 75 neurons) analyzed per well. Twelve wells from two independent cultures were analyzed for controls, Li-exposed controls, and 0.5 g/mL Au exposures; eight wells from these cultures were analyzed for 0.1 μg/mL Au exposures and 0.05 μg/mL Au exposures. To combine data from independent cultures, each well is typically normalized by the control wells for that individual culture (average of six wells). The two cultures used here had raw control values (mean ± standard error of the mean) of (284 ± 10) μm/and (281 ± 12) μm/cell. Since these values matched closely, we reported raw values for all treatment groups.

β-III-tubulin positive neurites extending from a cell body were considered positive neurites. Processes extending from the cell soma or from another neurite were classified as neurites when their length was equal to or greater than...
the diameter of the cell soma. Branch points identify secondary neurites, which originate from primary neurites, and tertiary neurites, which originate from secondary neurites, and so on. Branch points were scored for each cell without tracking the hierarchy associated with these points. Twelve wells from two independent cultures were analyzed for controls, Li-exposed controls, and 0.5 µg/ml Au exposures. For each treatment group, more than 1000 individual cells were scored. Cells were not scored if a primary neurite originating in the image perimeter (within one cell body of the image edge) extended beyond the image without branching. Therefore, a slightly smaller number of neurons were scored here than were utilized for total neurite outgrowth measurements. Example images show neurons and astrocytes, neurite traces, and neurite identification (Supplementary Information, Fig. S1).

2.10. Statistical analysis

All statistical analysis was performed with InStat (GraphPad Software). Each well was considered an individual sample, and mean values are reported plus or minus standard error of the mean. Student’s t test was employed for comparisons between controls and Li-exposed controls. For comparisons between controls and Au-exposed treatment groups, means were compared by one-way analysis of variance (ANOVA). For all comparisons, p < 0.05 was considered significant.

3. Results

3.1. AuNPs agglomerate slowly during incubation

Gold nanoparticle reference materials were characterized for size by multiple techniques in dry form and liquid suspension, are endotoxin free, and were provided in sealed ampoules. The dimensions indicated by these analytical techniques are smaller than the nominal size for the 30 nm and 60 nm reference materials (up to 17% and up to 11%, respectively). We first examined freshly dispersed 60 nm particles, since their intensity size distribution is not dramatically altered by excess serum proteins in solution (Tsai et al., 2011). When freshly dispersed at 5 µg/ml in water, the monomodal intensity size distribution (Z_\text{avg} = 55.8 nm) was within the reported uncertainty of the reference value. After three days incubation, the intensity size distribution was still monomodal, but had increased (Z_\text{avg} = 190.3 nm). When dispersed at this concentration in cell culture medium without serum proteins, the particles immediately agglomerated (Z_\text{avg} = 149.1 nm) and were not measureable after three days incubation. In cell culture medium with serum proteins, fresh dispersions had a monomodal intensity size distribution (Z_\text{avg} = 85.3 nm) that was relatively unchanged after three days incubation (Z_\text{avg} = 90.3 nm).

We next examined 30 nm particles, which were utilized for all subsequent experiments. When freshly dispersed at 5 µg/ml in cell culture medium with serum proteins, these particles agglomerated to a small extent (Fig. 1) despite the vortexing protocol, and very gradually increased in size during an incubation period of ten days. Although the intensity size distributions shown here are representative, the experiment was repeated five times. For these measurements, the dominant peak within the intensity size distribution (approximately 90%) indicated an agglomerate size of 120 nm, with a standard deviation of 30 nm. After three days incubation, the dominant peak was within 1% of the original value; after ten days incubation, it had increased by 50%.

Immediately after dispersion into cell culture medium with serum proteins, TEM images showed both individual particles and small clusters (Fig. 2a). Particles were located within a non-crystalline matrix, which is consistent with a corona formed by}

![Fig. 1. DLS intensity size distributions of AuNPs in differentiation medium containing serum proteins. Distributions were obtained within thirty minutes of dispersion (d0) and after 24 h (d1), 72 h (d3), 168 h (d7), and 240 h (d10).](image)
curves that encompassed typical signals from control cultures were measured without and with 0.5 μg/mL Au (Fig. 4). Standard curves were linear and there was no indication of particle interference.

In a separate experiment, NPCs were grown for ten days under control conditions. Freshly dispersed AuNPs were added to half the wells and all twenty wells were immediately assayed. Wells without particles had a value of \((1.84 \pm 0.02) \times 10^5\) RLU, whereas wells with 0.5 μg/mL Au had a value of \((1.79 \pm 0.02) \times 10^5\) RLU. These values are not significantly different from each other. For this experiment and the standard curves described above (Fig. 4), particles were added prior to the assay reagents, so both interference with the luciferase reaction and significant absorbance of the luminescent signal was ruled out. Cells cultured for ten days with nanoparticles (0.05 μg/mL, 0.1 μg/mL, or 0.5 μg/mL Au) or with 3 mmol/L Li\(^+\) were compared with controls (Table 1). No significant differences were observed, indicating that culture-level metabolism was not altered by these nanoparticles doses.

### 3.4. AuNPs do not alter neuron-specific outgrowth

After ten days in culture, immunocytochemical staining indicated the presence of both neurons (beta-III-tubulin positive) and astrocytes (glial fibrillary acidic protein positive) in all treatment groups (data not shown). Neurite outgrowth was evaluated within cultures exposed to non-cytotoxic doses of AuNPs, as demonstrated by culture-level metabolism (Table 1). Such doses did not significantly increase or decrease total neurite outgrowth per cell, primary neurites per cell, or branch points per cell (Table 2). Neurite outgrowth measurements were verified to be sensitive to the positive control, 3 mmol/L Li\(^+\), which reduced total neurite outgrowth per cell to approximately 65% of the length measured in control cultures. These measurements provide confidence that the culture was developing and responding normally, as this response has been demonstrated in multiple replicate cultures (Jeerage et al., 2012). Frequency analysis demonstrated that Li-treated control cultures had a greater fraction of cells with no branch points or one branch point compared to the other cultures, resulting in a lower mean value (Supplementary Information, Figs. S2 and S3).

For these studies, an individual chamber slide contained five wells seeded with cells, always arranged identically, with all treatment groups represented on a single slide. Comparing values determined within individual wells on each slide (Fig. 5) provides an additional way to evaluate the reproducibility of the rat cortex model’s response to nanoparticles or chemicals. For simplicity, only the highest nanoparticle concentration (0.5 μg/mL Au) was evaluated. Previous work showed that exposure to 3 mmol/L Li\(^+\) generated a reliable decrease in neurite outgrowth per cell, averaged over multiple technical and biological replicates (Jeerage et al., 2012). Here, individual Li-treated wells consistently contained neurons with less neurite outgrowth per cell, compared to control wells, whereas Au-treated wells were similar to control wells. The cross-slide coefficient of variation was 7.7% for Li-treated cultures, and 9.5% for 0.5 μg/mL Au-treated cultures. Similar analysis of branch points per cell showed greater variability, with a cross-slide coefficient of variation of about 15% for both Li-treated cultures and 0.5 μg/mL Au-treated cultures.

### 4. Discussion

Citrate ligands are commonly employed to stabilize nanoparticles during synthesis. Citrate stabilizes AuNPs electrostatically, via charge repulsion. In cell culture medium or other simulated extracellular fluids of high ionic strength, ions in solution shield the charges, reducing colloidal stability (Du et al., 2012). This was observed immediately when 60 nm AuNPs were dispersed. However, adsorption of serum proteins (e.g., bovine serum albumin) from the media can increase stability and prevent agglomeration. In particular, particles injected into medium while vortexing were rapidly coated with protein and remained near their primary particle size for two days (Zook et al., 2011). For studies with NPCs, a much longer window was required, as differences in neurite outgrowth may not be detectable after a short exposure period (Jeerage et al., 2012). Although the 30 nm AuNPs were added while vortexing, we obtained distributions larger than the primary particle size identified by measurements in water, likely due to the low serum content of differentiation medium. Interestingly, the 60 nm AuNPs also reached a similar agglomerate size, which has been
High polydispersity, evident in the DLS intensity size distributions (Fig. 1), indicates that the NPCs interacted with single particles, small agglomerates of 3–4 particles, and some larger agglomerates during their ten-day incubation. TEM images containing single particles (Fig. 2b and c) provided additional confidence. While only a small fraction of the particles could be evaluated by this technique, dropcasting and drying tend to induce agglomeration, so the presence of single particles is noteworthy.

Typically the uptake of metal nanoparticles is measured by inductively coupled plasma mass spectrometry. This technique can be used to compare differences in uptake due to nanoparticle size, shape, or surface chemistry at multiple timepoints. Its main disadvantage is that it is not known whether ions or particles are incorporated by the cells or whether the particles are internalized. In complex cultures such as the one employed here, results would be dominated by astrocytes and progenitor cells, which are the majority of cells found in this culture after ten days of differentiation (Jeerage et al., 2012). TEM is often utilized to provide this information, with unsurpassed detail on particle localization within thin (approximately 100 nm) cell slices. Although this approach provides superior resolution and contrast, sample preparation is complex. Furthermore, overall cellular morphology is sacrificed, which is not desirable in complex cultures with multiple cell types. Here, an alternative approach was employed, in which ion milling was alternated with particle detection to identify AuNPs in cell samples after minimal preparation.

FIB-milling of individual cells exposed embedded nanoparticles and nanoparticle clusters without altering the sample beyond the platinum coating and original dehydration process. By imaging the cross-sectioned cell in the HIM, we were able to observe exposed, but still-embedded, nanoparticles at a resolution sufficient to count and measure AuNPs. While this method does not provide the same resolution as TEM, the resolution of the HIM (compared to SEM), combined with the wider field of view, allowed us to characterize the distribution of AuNPs in the context of the entire cell body. Furthermore, the cell membrane and structure were preserved, giving, again, a more complete picture of nanoparticle location, which was consistently near the cell membrane. We chose cells for sectioning that had long processes suggestive of neurons, although we cannot definitely state that the endosomes containing AuNPs were located within neurons. We are currently developing multimodal imaging strategies to address these questions, which will be explored in a separate publication.

Our results conclusively demonstrate that changes in the culture environment or within cells due to 30 nm citrate-stabilized AuNPs do not significantly alter the metabolism of differentiating rat cortex neural progenitor cells or multiple measurements of neurite outgrowth after ten days of exposure. These results are consistent with studies in which mesenchymal stem differentiation was not altered by incorporation of AuNPs (Ricles et al., 2011). Interestingly, during the proliferation phase, similarly-sized (20 nm) AuNPs altered the growth characteristics of neural...
Table 2
Neurite outgrowth endpoints were determined after ten days exposure to Li+ or AuNPs in culture. Stars (**) indicate significant differences from control cultures (p < 0.0001); all other comparisons were not significant (p > 0.05). Total neurite outgrowth per cell was determined by automated tracing on an image-average basis. Primary neurites per cell and branch points per cell were scored manually. For the main treatment groups (Control, Li Control, and 0.5 μg/mL AuNPs), reported means and standard error of the mean (S.E.M.) are based on 12 wells from two independent cultures. Means for the remaining groups (0.1 μg/mL AuNPs and 0.05 μg/mL AuNPs) are based on 8 wells from two independent cultures.

<table>
<thead>
<tr>
<th>Chemical or nanoparticle exposure</th>
<th>Outgrowth per cell (μm)</th>
<th>Primary neurites per cell</th>
<th>Branch points per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>282 ± 7</td>
<td>2.83 ± 0.04</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>Li control (3 mmol/L)</td>
<td>185 ± 3***</td>
<td>2.81 ± 0.03</td>
<td>2.3 ± 0.1***</td>
</tr>
<tr>
<td>0.05 μg/mL AuNPs</td>
<td>280 ± 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 μg/mL AuNPs</td>
<td>269 ± 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 μg/mL AuNPs</td>
<td>267 ± 10</td>
<td>2.84 ± 0.05</td>
<td>3.1 ± 0.1</td>
</tr>
</tbody>
</table>

Overall, this work demonstrated that rat cortex neural progenitor cells exposed to biomedically relevant concentrations of gold nanoparticles (0.05 μg/mL Au, 0.1 μg/mL Au, and 0.5 μg/mL Au) did not exhibit altered metabolism or neurite outgrowth after ten days. Cells did incorporate citrate-stabilized gold nanoparticles dispersed in culture medium; helium ion microscopy images of cross-sectioned cells showed clusters of nanoparticles near the cell membrane in which individual nanoparticles could be identified and measured. For total neurite outgrowth measurements, slide-level comparisons indicated promising reproducibility in the response of these cells to both chemical (lithium) and nanoparticle (gold) assay controls, with a cross-slide coefficient of variation of less than 10% for both treatment groups. Our results suggest that 30 nm citrate-stabilized gold nanoparticles could serve as negative-control reference materials for in vitro neurite-outgrowth-based developmental neurotoxicity studies.

Funding information

This work was supported by internal funding at the National Institute of Standards and Technology (NIST).

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

Tammy L. Oreskovic was supported by a National Institute of Standards and Technology American Recovery and Reinvestment Act (NIST-ARRA) measurement science and engineering fellowship. Alexandra E. Curtain was supported by a National Research Council (NRC) postdoctoral fellowship.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2014.10.007.

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