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Characterizing Nanoparticle Dosimetry In Vitro

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Abstract

Nanoparticles, which are less than 100 nm in at least one dimension, are used in a variety of fields from engineering to health care. However, adverse effects of nanoparticle exposure are still under investigation. In vitro studies generally use high bolus doses that fail to emulate realistic effects on the human lung. The Air Liquid Interface (ALI) system models the effects of airborne nanoparticles on the lung by delivering aerosols to cells grown at the air-liquid interface. This may be superior to conventional in vitro exposure methods because it creates a more realistic nanoparticle deposition on the cell surface by minimizing overlying fluid and delivering the dose as an aerosol rather than a bolus. Initial experiments with the ALI system were performed using only RPMI culture medium in the wells to analyze gold nanoparticle (25 - 200 nm) deposition on the transwell membrane, and to test well-to-well variability. Once even deposition was confirmed, type I alveolar epithelial cells of the spontaneously immortalized R3/1 line were grown on transwells and utilized to determine effects following deposition of the nanoparticles. This cell type was used due to its usefulness as a target for nanoparticles, as type I squamous epithelium of the lung constitutes about 95-98% of the total available surface area. Scanning electron microscopy was used to visualize the deposited nanoparticles following exposure. LDH assays were conducted to measure cell response (cytotoxicity). The goal of this project is to compare the ALI nanoparticle delivery method to conventional exposure methods to determine if the latter overestimate or underestimate response. Future studies may include comparing the response from a constant dose of nanoparticles using the ALI system to the response generated using conventional exposure methods. These could lead to the development of highly accurate in vitro studies, which would exclude the use of animals.

Keywords: Nanoparticles, Dosimetry, In Vitro

1. Introduction

Nanoparticles (NPs) have unique physicochemical properties that allow for their use in various capacities. An increasing interest in the use of nanomaterials in multifarious processes has made the development of reliable methods of in vitro toxicity testing crucial for further progress in determining the safety of NPs. Thus, adverse effects of NP exposure are currently under investigation. The NACIVT (Nanoparticle Aerosol Chamber In-Vitro Toxicity) exposure system models the effects of airborne NPs on the lung by delivering aerosols to cells grown at the air-liquid interface (ALI)[1,8]. This may be superior to conventional in vitro exposure methods because it creates a more realistic NP deposition, or layering of NPs, on the cell surface by minimizing overlying fluid and delivering the dose as an aerosol rather than a bolus.

In this study, the NACIVT system was tested first with a gold NP aerosol and only cell culture medium in the wells to define a baseline amount of NP deposition. Gold NPs were used for these validation experiments because they are easy to measure analytically, and are also easy to visualize with Electron Microscopy (EM). In addition, they are relatively inert (toxicologically speaking). Thus, while they are not known to have any serious cytotoxicity, they are useful for method development. Deposition was then tested with 6.5 mm diameter, 0.4 μm pore size tissue culture

treated polystyrene Transwell® cell culture inserts on which rat type I alveolar epithelial cells of the spontaneously immortalized R3/1 line were grown at the air liquid interface. This was carried out to predict the effects of gold NPs on the lung.

Conventional in vitro exposures utilizing colloidal gold NPs were done as a comparison to NACIVT deposition. Once the exposures were completed, the media remaining was collected and analyzed to determine the amount of NPs in the cell pellet and supernatant. In addition to dosimetry measurements, lactate dehydrogenase (LDH) assays were conducted to measure cell response (cytotoxicity) and samples were processed for transmission electron microscopy (TEM). TEM was used to visualize the NPs that were taken up by cells during the exposures. The goal of this project is to compare the NACIVT NP delivery method to conventional exposure methods by characterizing dosimetry and cell response to determine if the conventional methods may overestimate or underestimate response.

2. Materials and methods

2.1 NACIVT Aerosol Delivery System

The NACIVT is an aerosol delivery system used to model the effects of airborne particles that are delivered to lung cells at the ALI. The NACIVT is controlled and monitored by a laptop that was pre-programmed with Labview software. Labview controls the electronics in the box below the deposition chamber and monitors temperature and relative humidity. The main screen displays all of the controls as well as the most important information on the operation of the chamber, including temperature and humidity setpoints, charger activity, electrometer voltage detection, and all temperature and humidity measurements. The fully assembled system begins to operate at a relative humidity (RH) of about 85% and a temperature of about 37° C. Once the temperature setpoints above and below the cells are reached, the metal well plate can be inserted and aerosol exposure can begin. The pump that takes in the aerosol is then turned on, as is the charger.

Once they are formed, the aerosolized NPs are negatively charged to promote electrostatic deposition on the NPs onto the cells. The NACIVT chamber pulls the aerosol at a flow of 1.7 lpm (liters per minute) out of a 30 L mixing chamber. The aerosol passes through a unipolar charger and is then split into two parts. The larger portion, about 1.1 lpm, is delivered to an electrometer that measures the current created by the charged particles when they are trapped in a filter. This current can serve to evaluate the particle concentration, which is useful in determining the similarity of the concentrations in different experiments[4]. The smaller portion, only about 600 mL/min, flows through the NACIVT well plate. Thus, the flow through each of the 24 wells is about 25 mL/min. The rest of the airflow leaves through exhaust systems. The smaller portion, or minor flow, passes through a humidifier that allows water molecules to pass through it and humidify the airflow.

The negatively charged NPs deposit on the apical (top layer) culture medium that overlies the cells (about 40 µL that cannot be completely removed due to capillary action through the Transwell® membrane) inside the Transwell® inserts that are placed in the metal well plate.

2.2 Cell Culture Model

Based on information by Koslowski and colleagues, it was determined that rat type I alveolar epithelial cells of the spontaneously immortalized R3/1 line from fetal rat lung were ideal for model development[5]. This is also supported by well-established predictive particle deposition models[3]. The alveolar epithelium of the lung will receive larger doses upon NP inhalation when compared to tracheobronchial and naso-pharyngeal-laryngeal epithelial cells and it constitutes about 95-98% of the total available surface area of the lung.

A rat cell line was used as opposed to a human cell line so that the data obtained could eventually be compared directly to results from rat in vivo NP inhalation studies conducted in the lab. These cells are grown at 37°C with 5% CO_2 in RPMI media with 10% heat-inactivated FBS (Fetal Bovine Serum) and 0.02 mg/mL gentamicin. The cells were grown in a polystyrene T75 cell culture flask, from which they were trypsinized and spun down, and plated in tissue culture treated polystyrene 24 well cell culture plates, Transwells®, or chamber slides as necessary. The time to confluency, or time for which cells were plated before being used in an experiment, was approximately 2 days.

The chamber slide functions by allowing cells to adhere to the bottom surface of the chamber slide, a glass microscope slide treated with a wash that encourages consistent growth on its surface. The cover of the chamber slide allows for gas exchange while simultaneously diminishing evaporation. Following incubation, the chamber can be

removed and the individual wells on the slide (separated by barriers) can be easily visualized by TEM. A polystyrene multi-well 24-well plate was used in the experiment for the LDH assay.

2.3 Mycoplasma Testing

Mycoplasma contamination can result in decreased cell viability, but cannot be observed with a microscope. Therefore, we used a test that measures the activity of mycoplasmal enzymes. Samples were collected and treated with lysis buffer, allowing any mycoplasmal enzymes to react with the MycoAlert® substrate (which contains luciferin and luciferase), which results in the conversion of ADP to ATP. The test reagent, which contains luciferase, then transfers the ATP into a light signal, and the ratio of ATP present before and after the addition of the substrate is calculated. The value of the ratio is used to determine whether or not the cells are contaminated with mycoplasma.

VenorGeM PCR Based Mycoplasma Detection Kit was also used to isolate and quantitate mycoplasmal DNA from cell cultures. Autoclaved DI water, Jumpstart Taq polymerase, and PurePak PCR microtubes were used in addition to the materials and reagents included in the kit. The Qiagen Dneasy protocol was followed in preparing samples for analysis by PCR to detect mycoplasmal DNA.

2.4 Aerosol Generation And Delivery To Cells At The ALI

Gold NPs were generated under Argon with a PALAS spark generator. First, the particles traveled to a 900 °C tube furnace as chain agglomerates and were vaporized. Upon exiting the furnace, they recondensed to form spherical NPs. They were then mixed in a 30 L chamber that had incoming flows of 5 lpm Ar from the aerosol generator, 2 lpm CO_2, 2 lpm O_2, and 25 lpm medical grade air, resulting in a total airflow of 34 L per minute.

The NP aerosol had a count median diameter of 66 nm with a geometric standard deviation of 1.4. The particle size was described with a count-based size distribution rather than a mass-based size distribution because the particles were too small to separate by mass. Upon deposition on Transwell® filters, agglomerates of up to 250 nm were visible (see below).

2.5 Conventional NP Exposures

The R3/1 cells used were passaged 13 times prior to the first use, while the cells used in the 4 h exposure were passaged 17 times prior to first use. A 100-µL volume of cells was added to 25 µL of trypan blue in order to determine percent viability and total cell count. The cells were counted using a hemacytometer. The cell suspensions used in the 4 h and 24 h exposures had a total cell concentration ranging from 696,875-978,125 cells/mL, with percent viabilities ranging from 93.7%-99.7%. Once the cell counts were determined, the volumes of cells and media for plating on the well plate and chamber slide were calculated. R3/1 cells were seeded into 24 well plates with 150,000 cells per well (300,000 cells/mL).

The colloidal gold (50 nm Gold Colloids purchased from BBI Solutions) was prepared as a suspension of NPs in cell culture medium containing serum, which coats the particles to minimize agglomeration. Agglomeration can be caused by attraction between the gold particles due to their neutral surface charges. The NP concentration of the colloidal gold stock solution was measured using atomic absorption spectrometry, and was found to be 46 µg/mL. The concentration was subsequently diluted to 40 or 10 µg/mL with complete cell culture media just prior to dosing the R3/1 cells. The NP concentrations used were 1, 5, 10, and 20 µg/mL.

The cells in the 24 well plates were exposed to NPs for either 4 hours or 24 hours, with exposures of each length conducted on three separate occasions. This was done to generate data with an n of for comparing length of exposure and observing trends in and consistency of results in terms of cell response. In some experiments, chamber slides were used for TEM imaging of cell uptake of NPs. R3/1 cells were seeded into 4 chamber slides wells with 135,000 cells per well (270,000 cells/mL), and the same procedure used for the well plates described above was used for the chamber slide except that the slide was exposed to gold NPs for only four hours, and the concentrations of gold particles used in dosing cells in each of the 4 sections of the chamber slide were 18, 9, 4.5, and 0.9 µg/mL. Chamber slide doses were calculated to match the gold concentration by surface area in the 24 well plates. In processing samples for TEM analysis, the cells were fixed with 2.5% glutaraldehyde and post-fixed in OsO_4, processed through a graded series of alcohols, infiltrated with liquid epoxy resin, and embedded on the glass surface with inverted capsular molds containing resin. After polymerization at 70°C, the hardened capsules containing the cells of interest were "popped-off" the surface of the glass slides. The "popped off" blocks were ultra-thin sectioned, stained using uranyl acetate, and lead citrate, and examined by TEM.

2.6 Cellular Dosimetry

2.6.1 air liquid interface

Initial experiments with the NACIVT were performed using only RPMI culture medium (without cells or Transwells®) in the wells to test well-to-well variability in gold
deposition. The well plate was exposed to the aerosol for 2 hours. Afterwards, the media from each well (about 450 µL) was pipetted into separate tubes for gold quantitation. In order to remove any remaining basal media from the wells, each well was washed twice with sterile deionized (DI) water, which was subsequently added to the tube. Once well deposition was evaluated, Transwells® containing cells were added to the wells to evaluate variability in deposition amongst all of the wells and to determine the effects of deposited gold NPs on the cells. Three different samples were collected and analyzed for gold content: apical medium, the polystyrene membranes with the R3/1 cells, and the basal medium.

2.6.2 conventional exposure

The same gold NP concentrations and cell culture conditions mentioned previously were used in the dosimetry experiments. After exposure (4 h and 24 h), the supernatant in each of the wells was collected individually. The wells were gently washed with 500 µL of HBSS buffer twice to remove any remaining particles, and the washes were added to the supernatant collection tubes of their respective wells (giving a total collected volume of 1.5 mL). In order to lift the cells off of the plate, 500 µL of 0.1% trypsin was added to each well and the plates were incubated for 15 min. The pellet fractions were collected in separate tubes. The wells were then washed vigorously twice with another 500 µL of HBSS buffer, and the washes were combined with the pellets, resulting in a total volume of 3 mL.

The dosimetry data collected shows the amount of gold taken up by or tightly associated with the cells versus that which was not taken up (the supernatant). The untreated cells had no gold in them, as expected, and both the supernatant and pellet had upward trends of gold measurements from wells 2 to 5, which contained increasing amounts of gold from a minimum of 1 µg/mL to a maximum of 20 µg/mL, respectively.

2.6.3 analytical methods

Gold determinations were performed using a Perkin-Elmer PinAAcle 900Z atomic absorption spectrophotometer equipped with longitudinal Zeeman background correction and a transverse heated graphite furnace (Perkin-Elmer Life and Analytical Sciences, Shelton, CT 06484 USA). Gold absorption was measured at 242.8 nm using a Perkin-Elmer Lumina hollow cathode lamp source. Gold was determined using non-linear regression analysis with known gold standards ranging from 5 ng/mL to 20 ng/mL. The apical and basal media samples were prepared by wet ashing the samples with ultrapure nitric and hydrochloric acid (2:1; SCP Science, Champlain, NY) at 90 °C. The membrane + cells were prepared for analysis by placing the Transwell® in 4% hydrochloric acid overnight. The range of the limit of detection was 0.0415-0.617 µg/mL, and the range of the limit of quantitation was 0.152-2.057 µg/mL. The quantitation of gold took into account all dilution volumes.

2.7 LDH Assay

The LDH assay is conducted to measure cell membrane integrity by measuring LDH activity following release into the cell culture medium from damaged cells. Thus, LDH release is an indicator of cytotoxicity. In this case, such a result would imply that the gold NPs are exceptionally harmful to the cells and may cause cell death. However, as discussed below, this was not expected to be the case, as gold is relatively inert and nontoxic. The procedure measures enzyme reaction rate, which has greater accuracy in determining how much LDH is contained within the sample.

After incubating the R3/1 cells for the times and at the gold NP suspension concentrations previously described, the media was aspirated from each well. LDH assay reagents included phosphate buffer, pyruvate, and β-NADH. The decrease in absorption of NADH and release of LDH was measured with the Agilent 8453 UV-visible Spectroscopy System (Agilent Technologies, Santa Clara, CA). LDH activity is proportional to the oxidation rate of NADH at 340 nm.

3. Results and Discussion

3.1 Mycoplasma Testing

The results of the mycoplasma test showed that the R3/1 cultures were not contaminated with mycoplasma.

3.2 Gold NP Deposition and R3/1 Cell Uptake

3.2.1 deposition in NACIVT system

EM was used to examine the gold NP distribution in the NACIVT wells following aerosol exposures. In the experiments where deposition was tested on bare Transwell® inserts, we found a fairly even gold NP distribution. The distribution shown below (Figure 1A) appears similar to another image taken at a lower magnification (Figure 1B), thus showing consistency of deposition. However, there were some outliers including one well that showed no deposition at all.

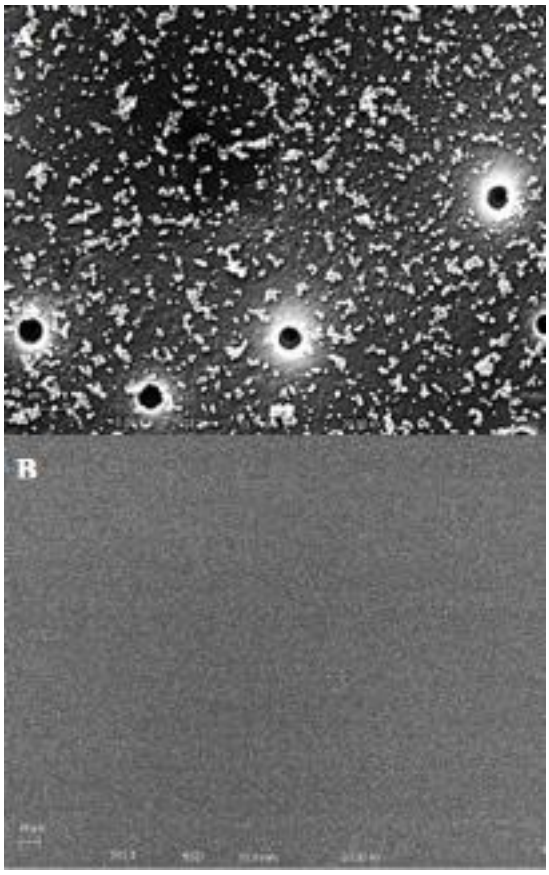

Figure 1. A. Transwell® containing media without cells. Gold NP deposition is shown at a magnification of 10,000 X, showing an ideal, even distribution of NPs. B. Transwell® containing media without cells, magnification of 200 X, showing an overall even distribution of gold

Once deposition among the wells (with only culture media) was analyzed, a subsequent experiment in which Transwells® with R3/1 cells were placed inside the NACIVT well plate exposed to gold NPs for 4 hours. The Scanning Electron Microscopy (SEM) images showed varying deposition among the wells of the well plate, with some wells obtaining an even distribution of gold NPs while others received little to no gold. These observations are

likely due to either uneven deposition on the wells themselves or to NPs and serum proteins that are present in the overlying cell culture medium. It also appeared that the gold NPs formed large agglomerates with which the cells were not interacting (Figure 2), at least over the time scale of this experiment. In addition, this variability could be due to the fixation and sample processing for SEM.

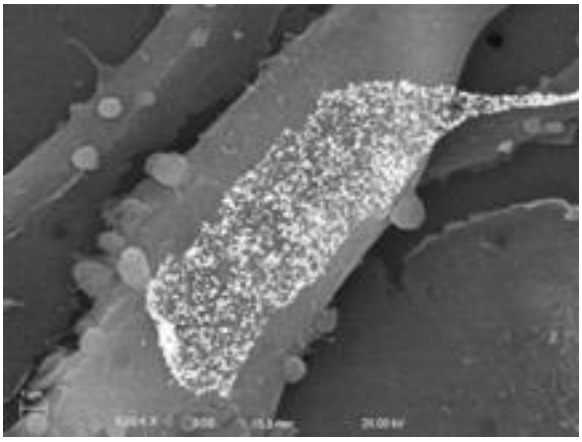

Figure 2. Transwell® with cells immediately fixed after gold aerosol particle deposition at the ALI

3.2.2 deposition via conventional in vitro exposure

TEM analyses of gold NP intracellular disposition following conventional exposure, shown in Figure 3, display a distribution of gold NPs throughout the cytoplasm (Figure 3A). The higher magnification images, for example Figure 3B, provide clearer views of the distributions of the gold NPs, showing that they can traverse the cell either alone or as agglomerates. Images depicting the nucleus such as Figures 3C and 3D, indicate that gold NPs are being excluded this subcellular compartment. Other images show NPs within intracellular structures. It is hypothesized that these

structures may be lysosomes, which are engulfing the NPs in order to destroy them because the NPs are foreign (and potentially toxic) to the cell, thus warranting removal from the cell.

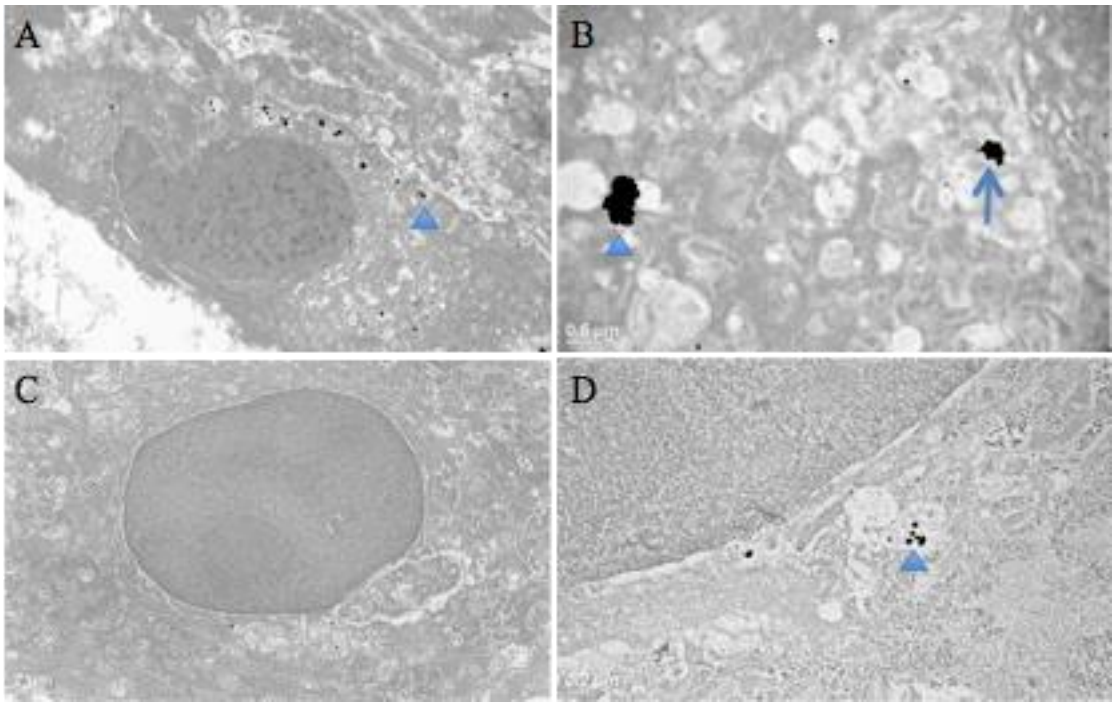

Figure 3. Intracellular gold NP distribution following conventional exposure. A. Perinuclear accumulation of gold NPs. B. Gold NPs appear as agglomerates (triangle) as well as singlets (arrow). C, D. Gold NPs are excluded from the nucleus

3.2.3 quantitation of deposited gold dose using NACIVT system

The experiments were conducted with and without cells in order to test for even deposition on the Transwell® insert membrane. Exposures without cells or Transwells® were conducted to determine if an even dose of gold NPs could be deposited around the annulus. The data obtained from the NACIVT exposures showed high well-to-well variability and a lack of consistency in order of deposition in terms of which wells received high deposited masses. For example, in experiments 1 through 4, wells 23, 1, 9, and 8 received the greatest amount of gold: 1367, 1841, 1684, and 1844 ng, respectively. The data obtained from these experiments is summarized in Table 1. The inconsistency in ranking of wells in terms of amount of deposited gold indicated that the nozzles of the system are not clogged, as no well was consistently receiving a low amount of gold NPs. The purpose of the experiment shown by the data in Table 2 was to measure the compartmentalized deposited dose (i.e. apical vs. basal medium and cell-associated). The total recovered gold, as shown in Table 2 is 469.5 ng ± 64.6.

Table 1. summary data: gold NP aerosol deposition using the NACIVT system without cells or Transwells® for 4 independent experiments (total of 24 wells)

Experiment	1 (10.1.14)	2 (10.8.14)	3 (10.29.14)	4 (12.10.14)
Mean (ng Gold)	549.5	1073	1330	653.2
St Dev	413.9	512.2	331.5	596.7
% Deviation	75%	48%	25%	91%

Table 2. gold NP aerosol deposition using the NACIVT system with cells and Transwells® in 24 wells

Experimental Samples	Apical Media	Basal Media	Membrane + Cells	Total
Mean (ng Gold)	432.2	28.76	8.58	469.50
St Dev	77.78	16.36	3.21	64.64
% Deviation	18%	57%	37%	14%

3.2.4 quantitation of deposited gold dose using conventional in vitro exposure

The results of the R3/1 exposure to 50 nm colloidal gold following 4-hour (Figure 4) and 24-hour (Figure 5) conventional exposures show an upward trend in the measure of gold delivered at successively higher gold NP concentrations, as expected. The percentages included in the graph represent the fraction of the total delivered dose that was cell associated. The amount of gold in the supernatant fraction and the pellet fraction should add up to the total delivered dose. For instance, the sum of the two rightmost columns (20 µg/mL) in green and blue should theoretically be equal to 20 µg. However, this is not seen due to the fact that gold NPs in media are lost in the system (e.g., media becomes stuck to, and is not retrieved from, the wells of the culture plates or in the pipette tubes used to transfer the media from the wells to dosimetry tubes. In comparison with the results of the dosimetry analyses following 24 h exposures, those following 4 h exposures (Figure 3) show higher percentages, or mean pellet fractions, in all 4 columns. For example, the mean pellet fractions following the 4 h exposures range from 8.6-34%, while those following the 24 h exposures range from 8-23%. This was more striking at the two higher concentrations of 10 and 20 µg/mL, at which gold NPs may form larger agglomerates.

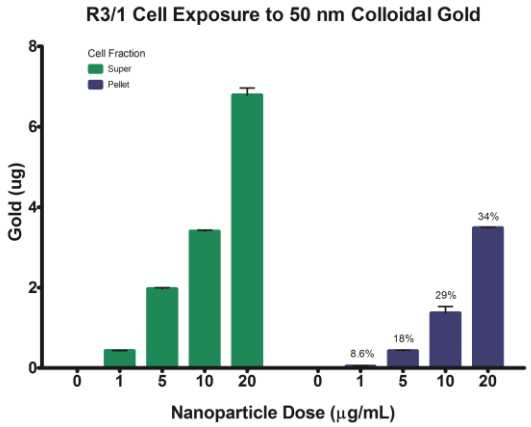

Figure 4. Measure of cell gold uptake following 4 h exposure n=3. Graphs show standard deviation. Percentages indicate the pellet fraction of the total dose delivered. Means ± SEM

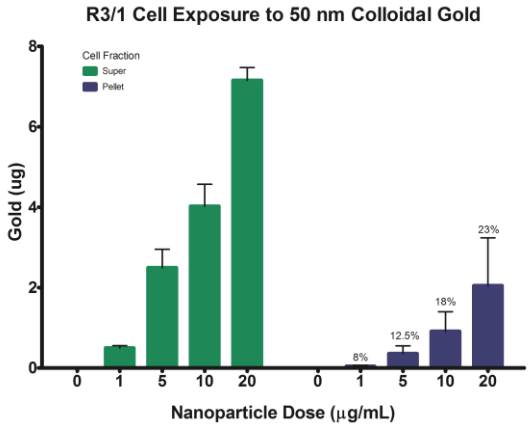

Figure 5. Measure of cell gold uptake following 24 h exposure n=3. Graphs show means ± SEM. Percentages indicate the pellet fraction of the total recovered dose (supernatant + pellet)

3.3 Cell Health Following Gold NP Deposition

3.3.1 exposure via NACIVT system

Figure 1 displays the results of an experiment comparing LDH activity in the supernatant of untreated R3/1 cells to that of cells exposed to gold NPs at the ALI for 4 hours. Culture medium was collected immediately after exposure, 24 hours after exposure, or 24 hours after exposure with fresh medium added. The 24 hour samples contained media transferred from the exposure as well as fresh media added to achieve the correct apical and basal volumes of 70 and 200 µL, respectively. In the 24 hour samples with fresh media, the media from the exposure was removed and replaced with fresh media in order to show the LDH activity retained in the original apical and basal media used in the experiments. The LDH activity in the apical and basal portions of the well were measured in order to show LDH release in the two compartments and determine whether there are differences in activity. The cells were healthy, but had a small response immediately after exposure (green bars, Figure 6), possibly due to the conditions of the exposure. For example, some cell membranes may have been damaged by insufficient humidity, leading to evaporation of media and drying out of cell membranes. Future experiments could include exposing cells for 4 hours without gold NPs and comparing the air-treated cells to gold aerosol-treated cells to see if they have a resulting increase in LDH activity, and thus determine if results have to do with media evaporation due to cells being under stress. In addition, if media evaporation is a problem, the exposure period could be decreased to 2 hours and LDH results compared.

It is unlikely that temperature or CO_2 levels caused a problem because cells were quickly transported between the incubator and exposure system. Further, the system was preheated to 37° C with CO_2 levels maintained at 5%. The untreated, immediate (basal), 24-hour, and 24-hour cells with fresh media were at control levels.

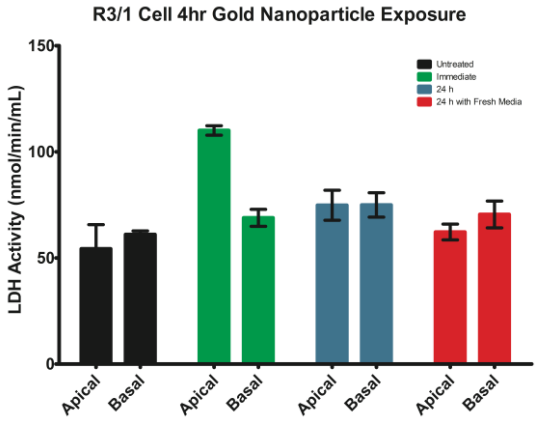

Figure 6. Gold NP exposure conducted at the air liquid interface comparing cells fixed immediately vs. at 24 hours. All graphs show means ± standard error of the mean (SEM)

3.3.2 conventional in vitro exposure

Figure 7 shows data from LDH measurements, which included samples from 4 hour and 24 hour exposures. The data shows that there is no dose dependent increase in LDH release, as this would likely be indicated by a large increase in absorbance[2]. There is also no interference of NPs with measurement. A drastic increase would indicate interference. There is also no increase in LDH release, indicating that the membrane integrity of the cells is not being compromised by the gold NPs and thus that the cytotoxicity of the NPs is negligible. This result is expected, as gold NPs are relatively inert[7]. Due to their non-cytotoxic nature, gold NPs are being investigated for use in a variety of settings including drug delivery. However, it is important for possible NP interactions to be studied further before combining gold with other materials in products designed for human use. For instance, it has recently been discovered that gold NPs can become toxic when coated with some charged molecules[6].

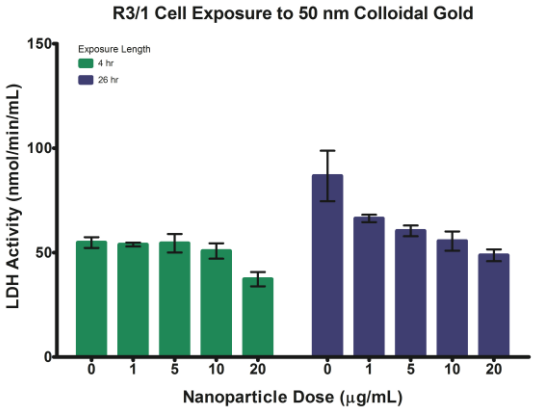

Figure 7. Graph of 4 h vs. 24 h LDH data n=3. All graphs show means ± standard deviation

4. Conclusions

Currently, one of the main barriers to immediate implementation of technology such as the NACIVT system for the purpose of replacing animals in biomedical research is the inaccuracy in delivered dose. Thus, the inconsistencies in exposure data must be corrected before the system can entirely replace in vivo exposure methods. Additional blank Transwells® for EM must be run in the NACIVT in order to ensure all of the wells are obtaining an even deposition of gold. Despite the high variability of gold NP deposition, current experimental results show a reasonable amount of deposition, with the data from the second exposure conducted showing deposition measurements twice as high as the first. Future experiments must be completed in order to determine how to better target the wells to ensure even

deposition within each well and among all of the wells around the annulus. In addition, further experimentation will be needed to validate the accuracy and consistency of NACIVT data in using other types of nanoparticles that are of greater interest (and which have higher toxicity) than gold (e.g. metal oxides). Such studies will have greater applicability for more common concerns involving NPs that consumers or individuals working with metals and other industrial materials could be exposed to at hazardous levels.

5. Acknowledgements

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6. References

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