Quantitative Assessment of Nanoparticle Induced Oxidative DNA Damage Using Isotope Dilution Mass Spectrometry

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ABSTRACT

Hyphenated mass spectrometry techniques have been employed as one of the primary analytical tools for investigating the effects of ionizing radiation, chemical/biological carcinogens, and oxygen derived free radicals on the induction and subsequent repair of oxidatively-induced DNA damage (DNA lesions) in living systems. The National Institute of Standards and Technology has established a comprehensive research program focused on identifying mechanisms of DNA damage caused by commercially relevant engineered nanoparticles (NPs) using high resolution mass spectrometry for the quantification of oxidatively-induced DNA damage. We present results from a recently published study on the genotoxicity of National Institute of Standards and Technology (NIST) standard reference material (SRM) gold nanoparticles using isotope dilution liquid chromatography /mass spectrometry analysis.

Keywords: nanotoxicology, Comet assay, genotoxicity, biomarker, toxicology

INTRODUCTION

In recent years, there has been substantial research interest in nanotechnology as a result of the unique or enhanced properties that many nano-scale particles exhibit. Nanoparticles are defined here as any particle that is less than 100 nm in any one dimension. With the maturation of this field and a greater understanding of the properties of these particles, there is increasing interest in the use of nanoparticles in consumer products. While research on the properties of nanoparticles for such applications will continue to increase, one of the limitations to the widespread application of nanoparticles is their potential human and environmental health effects. It is inevitable that nanoparticles will be released into the environment, and modeling efforts have begun to estimate the concentrations expected in different environmental matrices in the US and Europe [1-3]. What still needs to be understood is the extent to which these particles pose human or ecological risks resulting from their size-dependant properties.

One mode of action that is critical for determining how hazardous a chemical is to humans and organisms is genotoxicity, damage to the genetic material of cells or organisms arising from toxicant exposure. There are numerous components of genotoxicity such as the potential for gene mutations, chromosomal damage, and oxidative damage to DNA. This proceedings paper will focus on oxidative damage to DNA given that oxidative damage is one of the most widely acknowledged mechanisms of toxicity caused by nanoparticles [4]. Single cell gel electrophoresis (the COMET assay) is the most commonly used test for investigating genotoxicity; however, it is nonspecific and only yields an indication of total DNA damage, including oxidized purine base lesions, oxidized pyrimidine base lesions, abasic sites, and alkali-labile sites in a single number. Alternately, mass spectrometry (MS) based approaches such as liquid chromatography/mass spectrometry (LC/MS) and gas chromatography/mass spectrometry (GC/MS) have been used to quantify accumulated levels of individual DNA lesions [5-12]. This approach has substantial advantages over the Comet assay such as the potential for mechanistic understandings of the DNA damage process by comparing the relative levels of the different lesions measured. Additionally, lesion levels can be quantified by adding known amounts of stable-isotope labeled internal standards, thus yielding data that are traceable to standard reference materials that can be compared among laboratories to ensure the validity of the measurements.

This conference proceeding focuses on a recent study that determined the ability of nanoparticles to cause oxidatively-induced DNA damage in calf thymus DNA and cells [11]. The potential for National Institute of Standards and Technology (NIST) reference material (RM) gold nanoparticles (AuNPs) (10 nm, NIST RM 8011 - https://www-s.nist.gov/srmors/reports/8011.pdf; 30 nm, RM 8012 - https://www-s.nist.gov/srmors/reports/8012.pdf; 60 nm, RM 8013 - https://www-s.nist.gov/srmors/reports/8013.pdf) to damage DNA were studied using calf thymus DNA and HepG2 cells. The AuNP study utilized isotope-dilution LC/MS/MS to quantify 8-hydroxy-2'-deoxyguanosine (8-OH-dG), 8-hydroxy-2'-deoxyadenosine (8-OH-dA), (5'*S*)-8,5'-cyclo-2'-deoxyadenosine (*S*-cdA), and (5'*R*)-8,5'-cyclo-2'-deoxyadenosine (*R*-cdA) lesions. These nanoparticles were

thoroughly characterized with a range of analytical techniques in these published manuscripts [11].

METHOD

The following method description is modified from [11]. For preparation of test samples for the acellular study, 250 µL of a 500 µg/mL ct-DNA stock solution (prepared in distilled and deionized water (ddH_2O)) was added to a 1.5 mL Eppendorf tube and a specified volume of the appropriate AuNP RM solution + additional ddH_2O were added so that the final concentration of AuNP (gold atoms) in solution was (1, 100, and 10,000) nmol/L (molarity is with respect to Au atoms; alternatively, [0.0002, 0.02 or 2] µg Au/mL). For preparation of control samples, all sample additions were identical except that ddH_2O was added in place of the AuNP RM solutions. All test and control samples were prepared in triplicate. Samples were subsequently incubated at 37 °C for 4 h and then centrifuged at ~ 16,000 g for 60 min to pellet the AuNPs. Approximately 450 µL of the ct-DNA containing-supernatant was transferred into a 30 kDa molecular-weight-cutoff (MWCO) centrifugal filter unit (Millipore) and centrifuged at 7000 g for 15 min at 4 °C. After washing the filter membrane with ddH_2O, the ct-DNA was reverse-eluted into a clean 1.5 mL Eppendorf tube and the concentration of the eluted ct-DNA was determined using UV-Vis spectrophotometry.

Sixteen to 24 h prior to addition to cells, appropriately diluted AuNP RMs were pre-incubated (37 °C, 5 % CO_2) in culture media. Exponentially growing HepG2 cells (ATCC) were then cultured with the pre-incubated AuNPs for 3 h at 37 °C in the presence of 5% CO_2. The final concentration of AuNP in each culture was (1, 10, 100, and 1,000) nmol/L. Exposure of cells to 100 µmol/L hydrogen peroxide for 1 h was utilized as a positive control. All experiments were conducted in triplicate using independent T75 culture plates. At harvest, the supernatant was removed and cells were detached from each plate by first rinsing with Versene followed by incubation in 0.25% trypsin/EDTA. When cells showed signs of detachment, RPMI 1640 with FBS was added and cells were triturated thoroughly prior to being transferred to centrifuge tubes. Cells were pelleted at 1000 g for 5 min and washed twice using phosphate buffered saline (3.2 mmol/L Na_2HPO_4, 0.5 mmol/L KH_2PO_4, 1.3 mmol/L KCl, 135 mmol/L NaCl, pH 7.4) (Invitrogen). Cells and cell extracts were kept on ice for all remaining procedures. Genomic DNA was extracted from cell pellets using QIAmp Genomic DNA extraction kit (Qiagen) according to the manufacturer's instructions. The DNA pellet was thoroughly washed three times with ice-cold 70 % ethanol and once with ice-cold absolute ethanol. The absolute ethanol was removed from the pellet using a vacuum desiccator and the dry pellet was resolubilized (gentle horizontal shaking at 4 °C) in 120 µL ddH_2O for 24 h and the concentration determined.

The required volume of DNA from the cells or ct-DNA was transferred into a clean 1.5 mL Eppendorf tube so that the tube contained 50 µg DNA. The four internal standards ISTDs were added to the tube, the sample was dried in a SpeedVac under vacuum and then stored at 4 °C until enzyme digestion.

DNA samples (50 µg) were dissolved in 50 µL of a 10 mmol/L Tris-HCl solution (pH 7.5) supplemented with 2.5 µL of 1 mol/L sodium acetate containing 45 mmol/L zinc chloride (pH 6.0). Samples were incubated with nuclease P1, phosphodiesterase I and alkaline phosphatase for 24 h at 37 °C in a water bath as described previously [13]. The hydrolyzed samples were transferred into a 3 kDa MWCO centrifugal filter units (Millipore) and centrifuged at ~ 16,000 g (75 min, 4 °C). The filtrates were transferred into glass autosampler vials and analyzed by LC/MS/MS. Two separate LC/MS/MS analyses were performed on each DNA sample: one analysis for the hydroxyl-adduct lesions (8-OH-dG and 8-OH-dA) and one analysis for the tandem lesions (*R*-cdA and *S*-cdA) [14].

RESULTS AND DISCUSSION

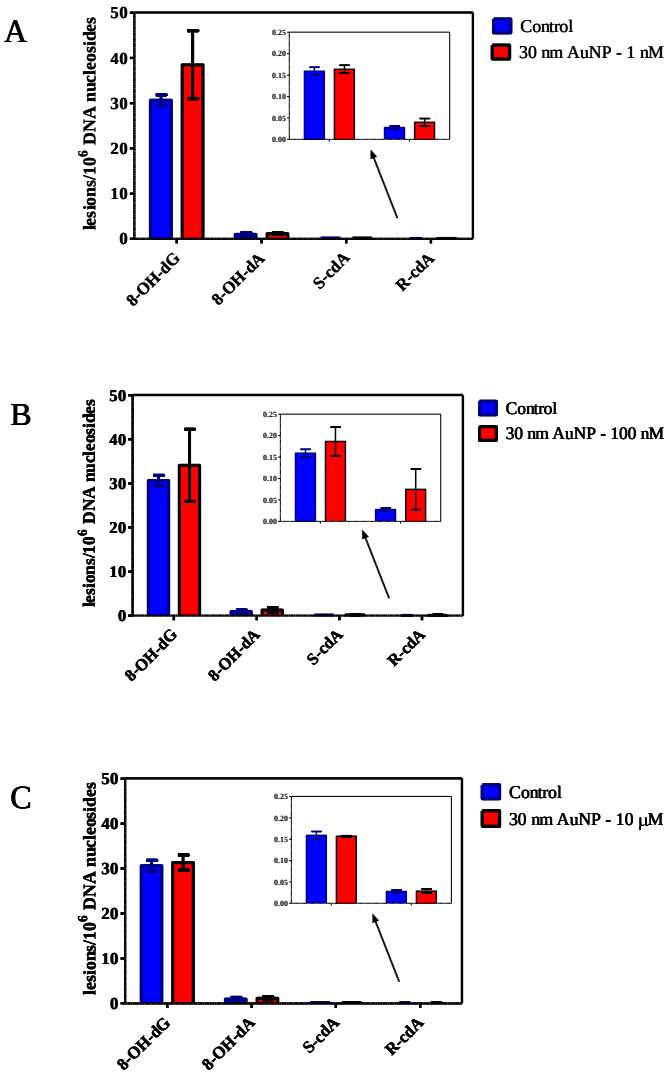
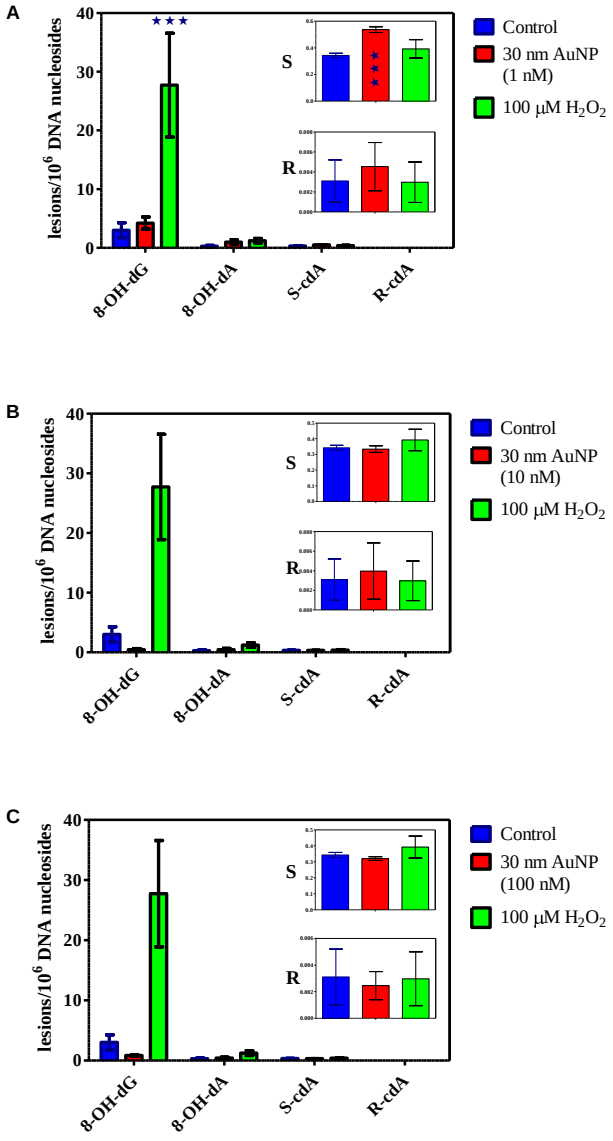


Figure 1: LC/MS/MS DNA damage evaluation of ct-DNA solutions (acellular system) dosed with NIST 30 nm AuNP RMs. (A) Measured lesion levels in the presence of 1 nmol/L AuNP. (B) Measured lesion levels in the presence of 100 nmol/L AuNP. (C) Measured lesion levels in the presence of 10 μmol/L AuNP. Blue: control lesion level. Red: experimental lesion level. The ratio of DNA lesions/10^6 DNA nucleosides represents the mean from three independent samples. The error bars represent standard deviations. Statistical analyses based on one-way ANOVA with posthoc Dunnett's multiple comparison test: * p value < 0.05; ** p value < 0.01; *** p value < 0.001. Reprinted with permission from [11].



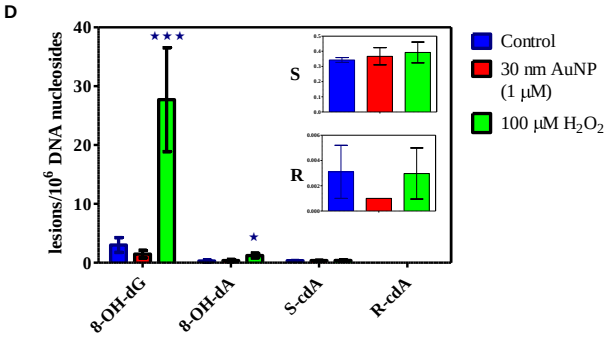


Figure 2: LC/MS/MS DNA damage evaluation of HepG2 cell cultures dosed with NIST 30 nm AuNP RMs. (A) Measured lesion levels in the presence of 1 nmol/L AuNP. (B) Measured lesion levels in the presence of 10 nmol/L AuNP. (C) Measured lesion levels in the presence of 100 nmol/L AuNP. (D) Measured lesion levels in the presence of 1000 nmol/L AuNP. Blue: control lesion level. Red: experimental lesion level. Green: positive control (H_2O_2) lesion level. The ratio of DNA lesions/10^6 DNA nucleosides represents the mean from three independent samples. The error bars represent standard deviations. Statistical analyses based on one-way ANOVA with posthoc Dunnett's multiple comparison test: * p value < 0.05; ** p value < 0.01; *** p value < 0.001. Reprinted with permission from [11].

The primary finding of this study was that NIST RM AuNPs did not cause elevated levels of the lesions studied at this range of AuNP concentrations. While elevated levels of *S*-cdA were observed for the lowest AuNP concentration, these results were not observed at higher concentrations suggesting that the result at the lower concentration is likely not reproducible. The results shown in Figures 1 and 2 are for the NIST 30 nm AuNPs, but similar results were obtained for the 10 nm and 60 nm AuNP RMs. Additionally, similar results indicating a lack of genotoxicity were obtained after exposing HepG2 cells for 24 h. The concentration range utilized was chosen to span that which could be used for biomedical applications of AuNPs such as for bioimaging. Thus, these results bode well for the potential application of AuNPs for treatment purposes. Moreover, there is a need for negative nanoparticle controls in nanotoxicology studies. Given the lack of genotoxicity and cytotoxicity observed in this study, these RM AuNPs could potentially fulfill this role given that they have been rigorously characterized and are available to laboratories worldwide with the guarantee of the same particles being delivered across a multiple year period.

We have many ongoing research projects designed to investigate different aspects of NP-induced oxidative DNA damage to isolated DNA, cells, and organisms. One of these projects relates to the potential for carbon

nanotubes to cause lesions to AML 12 cells; these cells are being exposed to different nanotube concentrations and for different durations related to the amount of time it takes for the carbon nanotubes to enter the cells. Another new research direction is utilizing *Caenorhabditis elegans* to assess the extent to which oxidatively-induced lesions are caused by silver nanoparticles or silver ions released by the nanoparticles. Calf thymus is also being used to determine the mechanism by which silver nanoparticles may induce DNA damage. Lastly, several projects are investigating the genotoxicity of a standard reference material nanoscale titanium dioxide (TiO_2). One project examines the potential of dispersed nanoparticles to cause oxidatively-induced DNA lesions to calf thymus DNA under various lighting conditions, while another looks at TiO_2 NP toxicity and uptake into food crops.

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