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

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ABSTRACT

Mass spectrometry techniques have been employed as one of the primary analytical tools for investigating the effects of oxygen-derived free radicals on the induction and subsequent repair of oxidatively-induced DNA damage in living systems. The National Institute of Standards and Technology (NIST) has established a comprehensive research program focused on identifying mechanisms of DNA damage that could be caused by commercially relevant engineered nanoparticles (NPs) using high resolution mass spectrometry for the quantification of oxidatively-induced DNA damage. We present results from recently published studies on the genotoxicity of NIST reference material (RM) gold nanoparticles (AuNPs) and on DNA damage in *C. elegans* after exposure to 10 nm silver nanoparticles (AgNPs).

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 has all three dimensions between 1 nm and 100 nm [1]. 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 [2-4]. What still needs to be understood is what measurements to collect to assess if these particles pose human or ecological risks resulting from their size-dependant properties.

One mode of action that is critical for determining if a chemical may be hazardous 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 oxidatively induced damage to DNA given that this type of damage is often observed in nanotoxicity studies [5]. 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 modified purine lesions, modified pyrimidine 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 [6-14]. 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 test conclusion.

This conference proceeding focuses on two recent studies. In one study, the ability of nanoparticles to cause oxidatively-induced DNA damage in calf thymus DNA and cells was assessed using NIST RM 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) was measured [12]. The AuNP study utilized isotope-dilution liquid chromatography tandem mass spectrometry (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. In the second study, the impact of AgNPs on the organism *Caenorhabditis elegans*

(*C. elegans*) was studied [14]. The AgNP study utilized isotope-dilution gas chromatography tandem mass spectrometry (GC/MS/MS) to quantify nine lesions: [4,6-diamino-5-formamidopyrimidine (FapyAde), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), 8-hydroxyguanine(8-OH-Gua), 8-hydroxyadenine (8-OH-Ade), 5-hydroxycytosine(5-OH-Cyt), thymine glycol (TG), 5-hydroxyuracil (5-OH-Ura), 5-hydroxy-5-methylhydantoin (5-OH-5-MeHyd), 5-hydroxymethyluracil (5-OH-MeUra)].

METHOD

NIST AuNP interactions with DNA were analyzed by the following method modified from [12]. 250 µL of a 500 µg/mL ct-DNA stock solution (prepared in distilled and deionized water (ddH$_2$O)) was added to a 1.5 mL Eppendorf tube and a specified volume of the appropriate AuNP RM solution + additional ddH$_2$O were added so that the final concentration of AuNP 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). These nanoparticles were thoroughly characterized with a range of analytical techniques as previously described [12]. For preparation of control samples, all sample additions were identical except that ddH$_2$O 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 $\approx$ 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$_2$O, 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.

The required volume of DNA from the 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 (*R*-cdA-^{15}N$_5$, *S*-cdA-^{15}N$_5$, 8-OH-dA-^{15}N$_5$, and 8-OH-dG-^{15}N$_5$) 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 [15]. The hydrolyzed samples were transferred into a 3 kDa MWCO centrifugal filter units (Millipore) and centrifuged at $\approx$ 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 8-OH-dG and 8-OH-dA and one analysis for the tandem lesions *R*-cdA and *S*-cdA [16].

For the investigation of the effects of AgNPs on *C. elegans*, the following abbreviated method modified from [14] was used. Worms were exposed in an axenic culture medium to 10 nm AgNPs (NanoCompsix, Inc., San Diego, CA) at concentrations of 10 µg/ml or 50 µg/mL, ionic silver (50 µg/ml) or iron II sulfide-nitrilotriacetic acid/ hydrogen peroxide (FeSNTA/H$_2$O$_2$) as a positive control. The AgNPs were previously characterized [14]. After exposure, DNA was extracted from the organisms by freezing and grinding the worms in liquid nitrogen and the DNA collected using kits from Qiagen. DNA pellets were then washed three times with ice cold 70 % (volume fraction) ethanol and once with ice-cold absolute ethanol to remove residual salts. DNA pellets were re-dissolved in distilled, deionized water (ddH$_2$O) and the DNA concentration was determined. Sample aliquots containing 50 µg DNA were prepared from each C. elegans extract and the ISTDs, FapyAde-^{13}C,^{15}N$_2$, FapyGua-^{13}C, ^{15}N$_2$, 8-OH-Gua-^{15}N$_5$, 8-OH-Ade-^{13}C,^{15}N2, 5-OH-Cyt-^{13}C, ^{15}N$_2$, TG-d$_4$, 5-OH-Ura-^{13}C$_4$,^{15}N$_2$, 5-OH-5-MeHyd-^{13}C,^{15}N$_2$ and 5-OHMeUra-^{13}C$_2$, d$_2$ were added to each sample. The samples were dried under vacuum and then stored at 4 °C prior to enzymatic digestion. Subsequent to enzymatic digestion, samples were dissolved in a buffer consisting of 50 mmol/L sodium phosphate, 100 mmol/L potassium chloride, 1 mmol/L EDTA and 100 mmol/L dithiothreitol (pH 7.4). To this solution, 2 mg each of *E. coli* formamidopyrimdine DNA glycosylase (Fpg) (Trevigen) and E. coli endonuclease (III) (Endo III) (Trevigen) were added and the sample was digested at 37 °C for 1 h. Hydrolysis using these enzymes prevents artifactual formation of DNA lesions because it only releases modified bases; consequently, there is no intact DNA base present during the trimethylsilylation step. The digestion was terminated with the addition of ice-cold absolute ethanol in combination with sample storage at –20 °C. Samples were centrifuged at 14 000 g for 30 min, supernatant fractions containing the excised DNA lesions were transferred to glass vials and the solvent was removed under Speed Vac. Samples were solubilized in ddH$_2$O, lyophilized, and then trimethylsilylated using bis(trimethylsilyl)trifluoroacetamide)/1% trimethylchlorosilane in pyridine (120 °C for 30 min). After derivatization, samples were analyzed by GC-MS/MS and the final results were reported in terms of the number of lesions quantified/10^6 DNA bases.

Statistical analysis of DNA lesions were conducted using Graphpad Prism software (Graphpad Software Inc., La Jolla, CA, USA). Outliers were removed using Grubb's outlier test and accounted for $\approx$ 5 % of the data collected. Data from different conditions were compared using ANOVA followed by Dunnett's test when multiple conditions were examined.

RESULTS AND DISCUSSION

The primary finding from the AuNP study was that NIST RM AuNPs did not cause elevated levels of the lesions studied at this range of AuNP concentrations. The results shown in Figure 1 are for the NIST 30 nm AuNPs, but similar results were obtained for the 10 nm and 60 nm AuNP RMs. 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 suggest that AuNPs would not have a genotoxic effect if they were used in biomedical applications. 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.
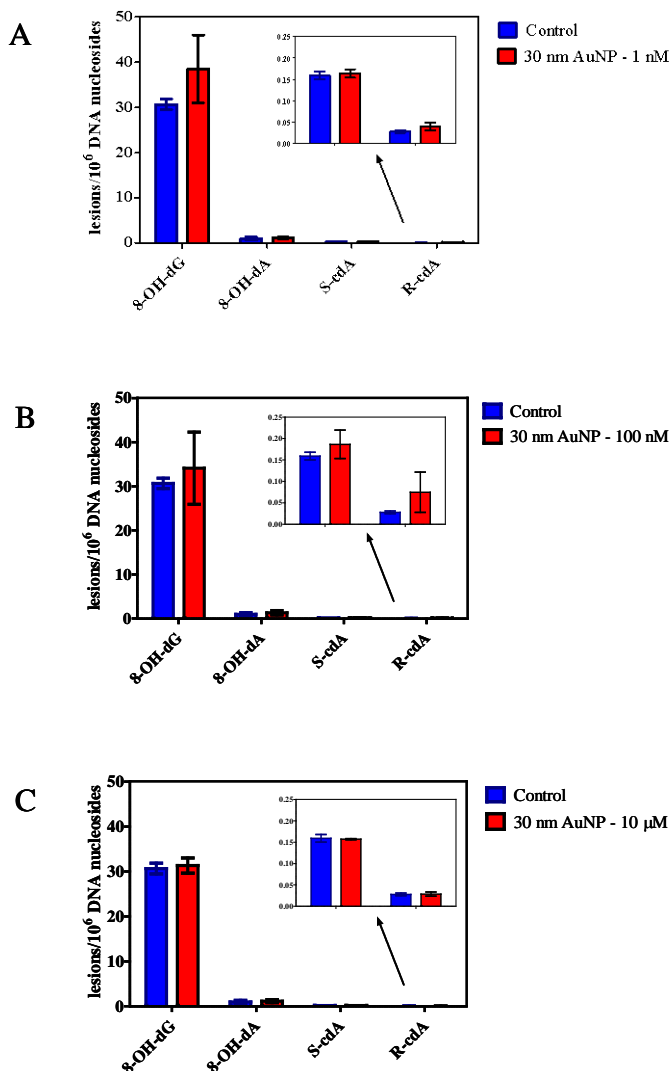
Figure 1: LC/MS/MS DNA damage evaluation of ct-DNA solutions (acellular system) dosed with NIST 30 nm AuNP RMs. (A) Measured lesion levels in the presence of 1 nmol/L AuNP. (B) Measured lesion levels in the presence of 100 nmol/L AuNP. (C) Measured lesion levels in the presence of 10 μmol/L AuNP. Blue: 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 [12].

In the investigation of the impact of AgNP exposure with *C. elegans,* statistically significant changes were only observed in the organism 8-OH-Gua levels. 8-OH-Gua levels were measured after exposure for 4 h or 24 h (see Figure 2). Iron II sulfide–nitrilotriacetic acid / hydrogen peroxide (FeSNTA/H_2O_2) reagent was used as a positive control for DNA damage and generated increased lesion levels at both 4-h and 24-h exposures.10nmAg exposure resulted in increased 8-OH Gua levels after 24 h at 10 µg/mL, but not the 50 µg/mL concentration, whereas silver ion exposure did not result in increased oxidative DNA lesion levels. Worms which were exposed to either 50 µg/mL 10nmAg or to 50 µg/mL Ag ions were experimentally shown to be growth inhibited. Therefore, we hypothesize that the DNA repair surveillance system may be upregulated in growth inhibited worms [17], resulting in reduced accumulation of 8-OH-Gua in the 50 µg/mL silver exposures.
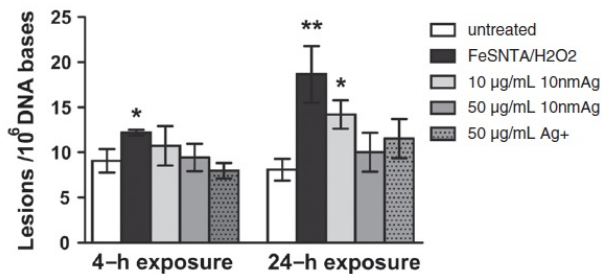
Figure 2. Formation of 8-OH-Gua in Caenorhabditis elegans exposed to 10nmAg. Asterisks indicate statistically significant results compared with the untreated samples using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. One or two asterisks indicate P<0.05 or P<0.01, respectively. All data represents the mean of six independent measurements. Uncertainties are standard deviations. Reprinted with permission from [16].

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. 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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