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# The Role of Surface Functionality on Acute Cytotoxicity, ROS Generation and DNA Damage by Cationic Gold Nanoparticles

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## Keywords

reactive oxygen species; gold nanoparticles; cytotoxicity; DNA damage; comet assay

Gold nanoparticles (AuNPs) are promising materials for biomedical applications [1,2] due to their tunable surface properties [3] and extraordinary stability.[4] Additionally, the inert core material reduces the potential for toxicity issues arising from particle degradation.[5] The size regime and concomitant geometrical outcomes including high degree of curvature, however, generates the potential for toxicity.[6,7] Generally, the toxicity of AuNPs depends on size, shape, the degree to which they aggregate, and their surface properties[8,9] Recently, several studies on the short-term cytotoxicity of AuNPs[10] and quantum dots[11]

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have focused on size,[12,13] shape,[14,15] and charge.[16] To date, however, issues such as ligand hydrophobicity have not been systematically explored.

In addition to acute toxicity arising from lysis and other disruptive events, DNA damage (genotoxicity) is an important issue in the application of nanomaterials. DNA damage provides both a useful strategy for anti-cancer drugs as well as a potential challenge for the design of non-cytotoxic therapeutics. Recently, El-Sayed and coworkers has shown that AuNPs with nuclear targeting motifs elicit significant damage of DNA and concomitant programmed cell death.[17] This outcome mirrors the behavior of anticancer drugs such as cisplatin and analogs.[18] As such, AuNPs can be envisioned as therapeutic agents in their own right as well serving as carriers for other drugs and biomolecules.[19] Taken together, an in-depth understanding of how NPs interact with cell surfaces and cellular organelles is central to their applications in biomedicine. [20]

Recently, several reports have described varying degrees of transfection efficiency of cationic lipids by increasing the chain length and hydrophobicity.[21] Hence, to examine the effect of surface hydrophobicity on acute and long-term nanoparticle cytotoxicity, we have synthesized a series of 2 nm core AuNPs that feature an quaternary ammonium functionality with a systematically varied (C1-C6) hydrophobic alkyl tail (Scheme 1). We have quantified the acute cytotoxicity of these AuNPs through mitochondrial activity assay, and the potential for long-term toxicity through quantification of reactive oxygen species (ROS) generation and DNA damage. Results are strongly dependent upon side chain structure, with greater acute toxicity and decreased DNA damage observed with increasing hydrophobicity. Significantly, these AuNPs can generate significant amounts of reactive oxygen species (ROS) that oxidatively damage DNA at concentrations that do not affect mitochondrial activity. These results suggest that caution is needed in the use of small AuNPs as carriers. Importantly, these results indicate the potential utility of these systems as cytotoxic therapeutic agents for cancer therapy.

The required AuNPs were synthesized via place exchange [22] of pentanethiol capped AuNPs (~2 nm) fabricated by Brust-Schiffrin reduction method.[23] Acute toxicity of the nanoparticles was determined through the alamar blue assay, a method based on mitochondrial activity. HeLa cells were treated with AuNPs dispersed in culture media with the concentrations ranging from 0–10 μM and incubated for 24 h. As shown in Figure 1, cellular viability decreases with increasing alkyl chain length, i.e. AuNP **1** is the least toxic (IC50 = 6μM) while AuNP **4** is the most toxic (IC50 = 0.71 μM).

In general, nanomaterials that retain >80% cell viability are considered safe for use in biological applications.[24] We next determined whether nanoparticles in this concentration range are capable of generating endogenous ROS and further causing DNA damage. As cellular uptake of these nanomaterials depends upon surface functionality,[25,26] we first used ICP-MS to determine the extracellular concentrations of nanoparticle required to obtain a constant intracellular AuNP concentration (214 ng/well, see supporting information). The calculated concentrations yielding 214 ng/well of intracellular gold were 100, 123, 148, and 165 nM for AuNP **1** to AuNP **4**, respectively, and were in the regime where treatment with all particles provided 100% viability.

Endogenous ROS production was quantified using 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA).[27] ROS species convert non-fluorescent H2DCFDA to its fluorescent 2 ′,7 ′-dichlorofluorescein (DCF) that can be quantified using a micro plate reader. Again, HeLa cells were treated with AuNPs dispersed in the cell culture media and incubated for 24 h. From Figure 2 it can be seen that the production of ROS was dependent on the AuNPs functionalization. The results indicate that increasing hydrophobicity

increases ROS production, a result that mirrors mitochondrial activity albeit at much lower concentrations.

Having established that hydrophobicity plays an important role in the generation of ROS and hence *potential* genotoxicity, we next explored the effect of particle hydrophobicity on DNA damage. DNA damage was quantified through the single cell gel electrophoresis, using the comet assay.[28] This technique provides a versatile and sensitive method to detect DNA damage. As with the ROS studies, the cells were incubated with AuNPs for 24 h. The cells were then trypsinized, embedded in agarose gel, and then lysed prior to performing gel electrophoresis. The DNA embedded in the gels was stained with SYBR Green dye and imaged by fluorescence microscopy (40X magnification) (Figure 3). The data were analyzed by Komet software. We observed that all four AuNPs significantly damage DNA in comparison to the control, as indicated by both % Tail DNA (Figure 4a) and Tail Length (Figure 4b). Interestingly, AuNP **1** caused significantly greater DNA damage than the rest of the AuNPs, contrasting with data derived from the mitochondrial activity (Figure 1) and ROS generation assay (Figure 2).

In addition, the percent of damaged cells was defined as those with values of % Tail DNA and Tail Length greater than two standard deviations above the mean of the control group values. Again, AuNP **1** resulted in the greatest percent of damaged cells (see Figure S4). The degree of DNA damage is comparable to mercaptoundecanoic acid-functionalized quantum dots (tail length~ 76±3.52 μm, @200μg/mL) [11] Notably, AuNP **1** causes DNA damage similar to cisplatin (79.7±8% DNA in Tail) when administered to cells at concentrations that yield 75% cell viability.[29]

In summary, we have determined that both the acute cytotoxicity and genotoxicity of positively charged AuNPs depend on the hydrophobicity of the ligands attached. Increasing the hydrophobicity of the particles increased their cytotoxicity. Increasing hydrophobicity likewise increased ROS production, even at AuNP concentrations where 100% cell viability was observed. Interestingly, DNA damage *decreased* with increasing particle hydrophobicity. In literature, conflicting cytotoxicity and DNA damage result has been reported between structurally similar complexes.[29] In addition, cells have *in vivo* mechanisms that maintain homeostasis.[30] Moreover, treatment with AuNPs induces the endogenous ROS production. This oxidative stress environment could initiate the autophagic process,[31] which can destroy foreign molecules to avoid cell death. This process may contribute cell survival in an oxidative environment of NPs. Therefore, AuNP 4 which produces more ROS, can be presumably degraded due to autophagy and will be less available to damage DNA. Taken together, these studies indicate that AuNPs can be employed not only as carriers but also as potential therapeutics that exploit their capability to elicit cell function and generate cytotoxic and genotoxic responses.

## Experimental Section

### Cell culture

HeLa cells were cultured at 37°C under a humidified atmosphere of 5% CO2. The cells were grown in low glucose Dulbecco's Modified Eagle's Medium (DMEM, 4.0 g/L glucose) containing 10% fetal bovine serum and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The cells were maintained in the above media and subcultured once every four days.

### Alamar blue assay

The cell viability was evaluated by using an alamar blue assay according to the manufacturer's protocol (Invitrogen Biosource, USA). In a typical experiment, cells were

seeded at 3000 cells/well in a 96 well-plate 24 h prior to the experiment. On the following day, the old media was aspirated and cells were washed one time with cold PBS before putting the different concentrations of AuNP **1**, AuNP **2**, AuNP **3** and AuNP **4** ranging from 0–10 μM dispersed in the pre-warmed serum-containing media. The cells were again incubated 24 h at 37°C under a humidified atmosphere of 5% $CO_2$. On the next day, after thoroughly washing the cells three times with PBS buffer, the cells were treated with 220 μL of 10% alamar blue in serum-containing media. Subsequently, the cells were incubated at 37°C under a humidified atmosphere of 5% $CO_2$ for 3 h. After 3 h of incubation, 200μL of solution from each wells was taken out and placed in a 96-well black microplate. Red fluorescence, resulting from the reduction of alamar blue solution, was valued (excitation/emission: 535 nm/590 nm) on a SpectroMax M5 microplate reader (Molecular Device) to determine the cellular viability. Each experiment was done in triplicate.

## Endogenous reactive oxygen species (ROS)

The ROS was determined through a microplate reader. HeLa cells were plated into a 24-well plate (20K /well) for 24 h prior the experiment. On the following day, cells were treated with AuNPs with the concentration of 100, 123, 148, and 165 nM for AuNP **1** to AuNP **4**, respectively. As a positive control experiment, cells were treated with 0.3% $H_2O_2$ suspended in culture media and incubate for 30 min at 37°C before performing the assay. After 24 h incubation, cells were wash with PBS 3 times and were subsequently treated with 2 ′,7 ′-dichlorodihydrofluorescein diacetate, H2DCFDA (Molecular Probes/ Invitrogen) (250 mL, 5 μM/well). Experiments were done in triplicate. After incubating for 30 min, the cells were washed by PBS and lysed with cell lysis buffer (Gene Therapy Systems). 200 μL of solution from each well was transferred to a 96-well black microplate. Fluorescence intensity, resulting from the oxidation of dye, was valued (excitation/emission: 488 nm/520 nm) on a SpectroMax M5 microplate reader (Molecular Device) to determine the level of ROS. The level of ROS production by AuNPs was compared using a one way ANOVA and post hoc t-tests with a Bonferoni Correction for multiple comparisons. All four AuNPs resulted in a significant increase in ROS levels (ANOVA; $F=45.08$; $df=5, 11$). Moreover, ROS produced by AuNP **1** was significantly less than AuNP **2** ($p=0.006$), AuNP **3** ($p=0.005$), & AuNP **4** ($p=0.0001$).

## Comet assay

For the comet assay, the HeLa cells were maintained as mentioned above. HeLa cells (20K cells/well) were plated in a 24-well plate for 24 h prior to performing the experiment. On the day of the experiment, cells were washed using cold PBS once. Thereafter, AuNPs were dispersed in pre-warmed serum-containing media with the final concentration determined according to ROS experiments. Cells were treated with AuNP solution and kept in cell culture incubator for 24 h. For the comet assay, cells were washed in PBS three times and trypsinized by trypsin EDTA 1X (MediaTech, Inc, USA). Cells were collected by centrifugation at 1000 rpm, 5 min. After centrifugation, cells were dispersed in PBS and embedded in 1% low melting point agarose (Agarose Type I-B, Sigma Aldrich). The agarose gel was transferred to CometSlide™ HT (Trevigen, USA) and kept in 4°C refrigerator for 10 min. Slides were placed in a cold lysis solution (2.5 M NaCl, 80 mM $Na_2$-EDTA, 1 mM Tris-HCl, and 1% Triton X-100, pH 10) and kept at 4°C in the dark for 24 h. Electrophoresis was performed in 0.3 M NaOH, 0.5 mM $Na_2$-EDTA, and pH 13 for 35 min at 20 V, 300 mA. The slides were then neutralized and stained with SYBR Green dye (Invitrogen, USA). The comet images were pictured for 34, 52, 59, 76 nuclei for AuNP **1–4**, respectively using a fluorescence microscope (40X magnification, Nikon E600 microscope stand) with a green filter. The data were analyzed through Komet software. One-way analysis of variance revealed significant effects of treatment group for both tail length ($F=10.33$; $df=243, 4$; $P<0.001$) and % tail DNA ($F=12.18$; $df=242, 4$; $P<0.001$). Post hoc t-

tests with Bonferoni Correction for multiple comparisons demonstrated that all of the nanoparticles caused significant DNA damage.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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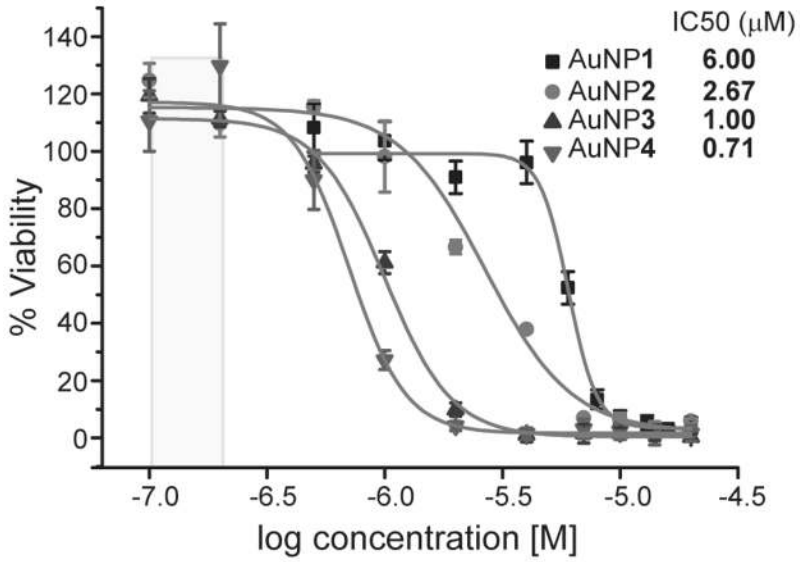



**Figure 1.**

Cytotoxicity of AuNP **1–4** in HeLa cells determined by alamar blue assay and $IC_{50}$ of particular AuNPs. The box represents the concentration range used in the ROS generation and DNA damage study.

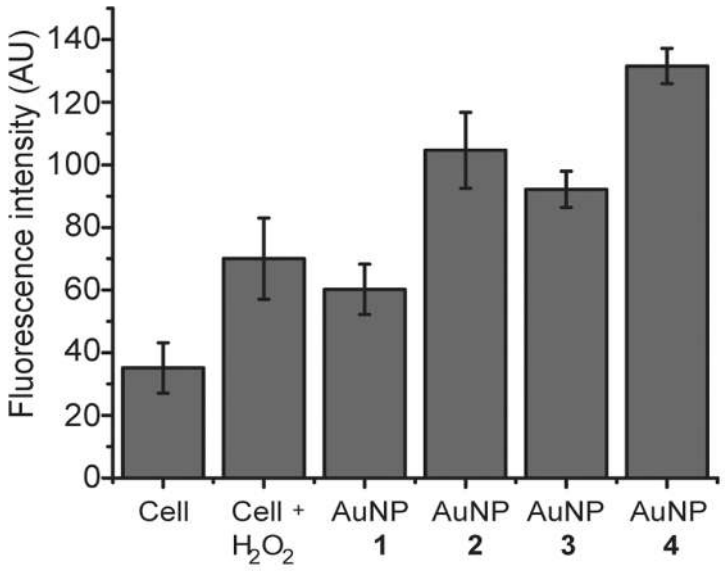



**Figure 2.**

Quantifications of ROS in HeLa cells determined by the oxidation of H2DCFDA dye. The intracellular gold in each AuNPs was 214 ng/well. The controls were cells alone and cells treated with exogenous H2O2 (0.3% v/v). The data were statistically analyzed and significant ROS level difference was found between AuNP **1** and AuNP **4** (t=12.57, p=0.0002)

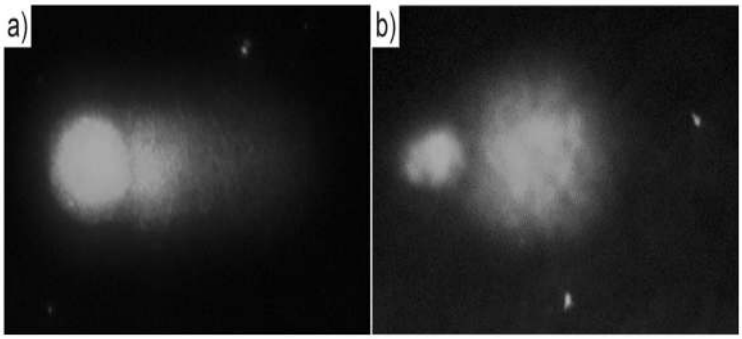



**Figure 3.**
Optical images of comet assay a) Cell alone, b) Cell treated with AuNP **1**

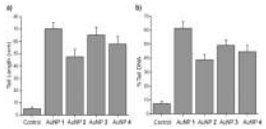
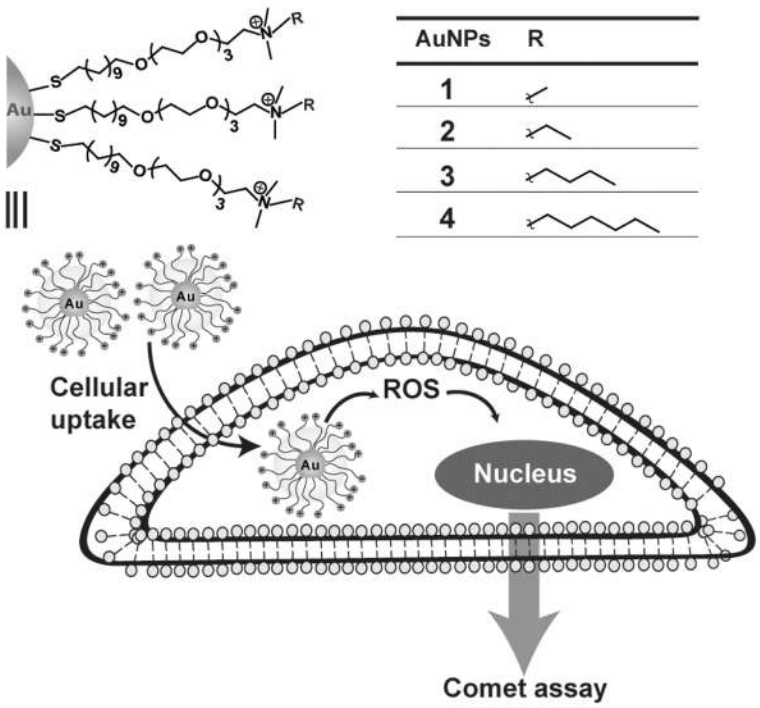


**Figure 4.**

a) Tail Length of AuNP **1–4** from the comet assay b) % Tail DNA of AuNP **1–4** from the comet assay. Statistical analysis by ANOVA and post hoc t-tests with Bonferoni Correction for multiple comparisons revealed all of the nanoparticles caused significant DNA damage. ($p<0.001$)




**Scheme 1.**
A series of AuNPs used in this study and a proposed mechanism of DNA damage determined by comet assay.