Cytotoxicity and Genotoxicity of Silver Nanomaterials

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ABSTRACT

Silver nanomaterials have great potential in industrial and medical applications but their potential impacts on health and environment remain unknown. In this study, we have investigated the cytotoxic and genotoxic effects of engineered silver naomaterials. Using the MTT assay, we compared the cytotoxicity of dialyzed silver nanoparticles (Ag NPs ;15 nm), polyethylene glycol coated Ag NPs (PEGylated Ag NPs) and silver nanorods on the survival of various human cell types including T cell leukemia JURKAT, B lymphoblastoid cells RAJI, human umbilical vein endothelial cells (HUVEC) and human peripheral blood mononuclear cells (PBMC). All these silver nanomaterials show cytotoxic effect but with various degrees, depending on their types, concentrations, treatment times and the target cell types. In general, silver nanorods appear to have the highest toxicity, with no survival in either JURKAT or RAJI cells following 24 h treatment at 0.2 ug/cm$^{2.}$ The potential genotoxic effect of Ag NPs was tested on human peripheral blood samples using the cytokinesis-block micronucleus (CBMN) assay. In comparison to untreated controls [with 0 – 30 micronuclei or MNi; a biomarker of chromosome breakage or loss) per 1,000 binucleated (BN) cells] human peripheral blood cells treated with 0.32, 1.6, 3.2, 16, and 32 ug/ml Ag NPs for 72 h exhibited 26.5, 58.5, 53, 64, and 44 micronuclei (MNi) per 1000 BN cells, respectively. The Nuclear Division Index (NDI; an indicator of cell viability and proliferation) of these Ag NP-treated blood cells were determined to be 1.52, 1.27, 1.27, 1.2, 1.1 respectively, with most values lower than the normal scores (1.3 to 2.2) found in untreated blood cells. Our results suggest that the toxic potential of engineered silver nanomaterials depends on their physicochemical properties, assay concentrations and incubation time as well as target cell types. The findings also demonstrate that silver nanoparticles could reduce survival and cause DNA damage in human peripheral blood cells.

Keywords: nanoparticles, , nanotechnology, silver nanomaterials, nanotoxicology, cytotoxicity, genotoxicity,

1. BACKGROUND

Nanotechnology is a multidisciplinary science and technology which involves the engineering and application of nanomaterials having at least 1 dimension with a size from 1 to 100 nm. As such, nanomaterials have proportionally larger total surface area, empowering them with mechanical, magnetic, electronic or color properties unachievable by same materials at larger size scales. These unique physicochemical properties endow the nanomaterials with tremendous application potential in industrial and medical production, driving the value of the nanotechnology industry to several hundred billion dollars [1,2,3]. The wide demand is projected to increase the production of nanomaterials to 58 000 tons by 2020. Because of their effective antimicrobial properties [4,5] Ag NPs have become one of the most commonly used nanomaterials in consumer products such as electronics and medical fabrics. Their current usages also include probes in cancer research and drug delivery in pharmaceuticals. Silver nanoparticles have also shown promise as catalysts for pollution control, chemical synthesis, and fuel cells. At present, little is known about the toxicological effects of many engineered nanomaterails including Ag NPs, resulting in a lack of regulation guidelines and increasing concern for their potential health and environmental impacts [1,2,3]. This research aims

to fill the knowledge gap by investigating the potential cytotoxic and genotoxic effect of silver nanoparticles. Because structure, size and chemical composition of nanoparticles play an important role in their reactivities , we have included in our study 3 different types of silver nanomaterilas, i.e., dialyzed sphere silver nanoparticles (15nm) , pegylated silver nanoparticles (15 nm), and silver nanorods. In the cytotoxicity studies we have studied the cytotoxic effect of the silver nanomaterials on 4 different human cell types using the commonly used MTT assay kit. To investgate the genotoxicity of silver nanomaterials we have focused on the effect of dialyzed silver nanoparticles (15 nm) on fresh human blood cells by conducting the well-established Cytokinesis-Block Micronucleus (CBMN) Assay [6].

2. METHODS

2.1 Chemicals and Reagents

The cell growth determination kit (MTT based) was purchased from Bioassay system. Phytohemagglutinin (PHA-P), Histopaque-1077-1, and cytochalasin B were purchased from Sigma-Aldrich (St.Louis, MO). Endothelial cell growth supplement was obtained from BD Bioscience (Bedford, MA). Gurr Buffer tablets were purchased from EMD (NJ). Giemsa stain was a product from Ricca chemical Company (Arlington, TX). Cytoseal 60 was purchased from Richard Allan Scientific (Kalamazoo, MI).

2.2 Cell Culture

Human Umbilical Vein Endothelial Cells (HUVEC) (ATCC number CRL-1730) were cultured in F-12K Medium with 0.1 mg/ml heparin, 0.03-0.05 mg/ml endothelial cell growth supplement (ECGS), and 10% fetal bovine serum (Omega Scientific). The B lymphoblast cell line RAJI (ATCC number CCL-86), human peripheral blood mononuclear cells (PBMCs, freshly isolated by Ficoll-Hypaque centrifugation of whole blood) and the Jurkat T leukemia cell line (ATCC number TIB-152) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% penicillin/streptomycin, and 1% Fungizone (Omega Scientific). All cultures were grown in a 37°C incubator with 5% CO_2. Freshly isolated human PBMCs were stimulated with PHA-P before use in cytotoxicity assay.

2.3 Silver Nanoparticles

The nanoparticles used were produced by Dr. Michael Mason's laboratory in the department of Chemical and Biological Engineering at the University of Maine. Silver nanoparticles **(**15 nm) were synthesized by reduction of silver nitrate with sodium citrate and dialyzed overnight to remove excess citrate. This synthesis followed a method similar to that described previously [e.g.7]. Silver nanorods were generated by surfactant templated growth using cetyltrimethylammonium bromide (CTAB). Silver nanoparticles were coated with hydroxyl-terminated PEG to prevent agglomeration [8]**.** The size and geometry (volume and surface area) of the nanoparticles were determined using a combination of TEM (Transmission Electron Microscopy), UV-visible absorption spectroscopy, dynamic light scattering and dark field micro-spectroscopy according to published methods. Before use in cell treatment, the dialyzed silver nanoparticles were sonicated using the Misonix Ultrasonic Liquid Processor (models XL-2000) while the PEGylated silver nanoparticles and the silver nanorods were centrifuged at 13,000 rpm for 10 min and resuspended into the original volume with water.

2.4 MTT Cytotoxicity Assay

Cells were seeded in a 96-well plate and cultured for 1 day (suspension cells) or 2 days (monolayer cells). During the log phase of growth cells were treated with silver nanoparticles, peglylated silver nanoparticles or silver nanorods at various concentrations (0.01, 0.05, 0.1, 0.2, 0.5, 1, 5 and 10 ug/cm^2) for 24 or 120 hours. Yellow MTT reagent was then added to react for 4 hours with the the mitochondrial dehydrogenase in viable cells to form blue formazan crystals. Finally, MTT solvent was added to dissolve the formazan crystals by shaking the plate at room temperature for 1 hr. The plate was read at 570 nm on a spectrophotometer (Bio-Tek Instruments) with a reference wavelength of 690 nm. The OD_{570nm} of the untreated control culture is considered total survival from which the relative survival values of the nanomaterials treated cultures are determined.

2.5 Cytokinesis-Block Micronucleus (CBMN) Assay

The CBMN assay was performed using whole blood cultures as described [6]. Typically, 0.5 ml of whole blood is added to 4.5 ml of complete medium (RPMI 1640) supplemented with the PHA mitogen. After 44 hr, the culture was treated with cytochalasin B at 6 mg/ml, a recommended concentration optimal for accumulating binucleated (BN) cells and harvested at the end of 72 h by centrifugation at 300 x g for 5 min. The cells were hypotonically treated with 7 ml of cold (4°C), 0.075 M KCl to lyse red blood cells and centrifuged immediately at 300 x g for 8 min. The cells were fixed in 5 ml of methanol:acetic acid (3:1), centrifuged, washed twice with the fixative, and resuspended in 0.1 to 0.2 ml fixative. The cell suspension was applied drop wise onto clean glass slides and allowed to dry. Staining of cells was carried out using Giemsa stain in Gurr's buffer. Slides were examined to determine mononucleated, binucleated (BN), multinucleated cell, BN cell containing one or more micronuclei (MNi), BN containing an nuclei plasmic bridge (NPB) as well as BN cell containing nuclear buds (NBUDs). The distribution of mononucleated, BN and multinucleated cells are used to determine nuclear division index (NDI). The frequency of BN cells with MNi, NPBs or NBUDs provides a measure of genome damage and/or chromosomal instability. According to the published report, the ranges of values for various nuclear events in normal control cultures are: BN cells: 30–60%, NDI: 1.3–2.2, MNi per 1,000 BN cells: 0–30, NPBs per 1,000 BN cells: 0–10, NBUDs per 1,000 BN cells: 0–5.

3. RESULTS

3.1 Cytotoxicity of Silver Nanomaterials

Dose-response studies using the MTT assay demonstrate that Ag NP treatment for 24 hr does not show significant toxicity at 1 ug/cm^2 or lower concentrations. Significant toxicity is seen at 5 ug/cm^2, with higher toxicity in JURKAT (no survival) and HUVEC (25 % survival) than in PBMC (65% survival) and RAJI (80% survival) (Fig. 1). By increasing treatment time to 120 hr, the Ag NPs began to show almost 100% toxicity in both JURKAT and RAJI even at a concentration as low as 0.2 ug/cm^2 (data not shown). PEGylated Ag NPs appeared to be less toxic, showing a lack of dose-dependent response over concentrations 0.25, 0.5, 1.0, 5 and 10 ug/cm^2. Overall it causes approximately 60 to 70 % relative survival in JURKAT when treated for 24 hr at these concentrations (Fig. 2). Again, prolonged treatment with PEGylated Ag NPs to 120 hr increases its toxicity, leading to 100 % toxicity in JURKAT even at 0.5 ug/cm^2 (data not shown). Among the 3 types of silver nanomaterials, silver nanorods appears to have the highest cytotoxicity. Treatment at 24 hr at 0.2 ug/cm^2 led to almost 100 % cytotoxicity in all the 4 different human cell types tested. (Figure 3). Increased treatment time to 120 hr also resulted in high toxicity at lower concentrations. (data not shown).

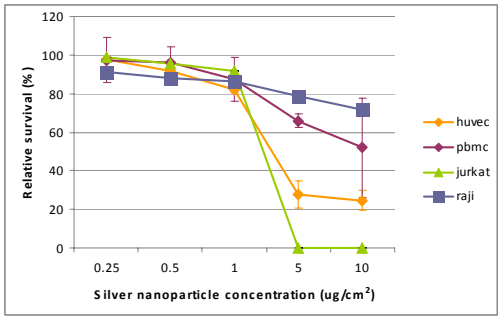

Figure 1. Cytoxicity of 15 nm dialyzed silver nanoparticle in human cells following 24 h treatment. Relative survival of Ag NP treated cells was determined by the MTT assay. Results show averages of 3 experiments

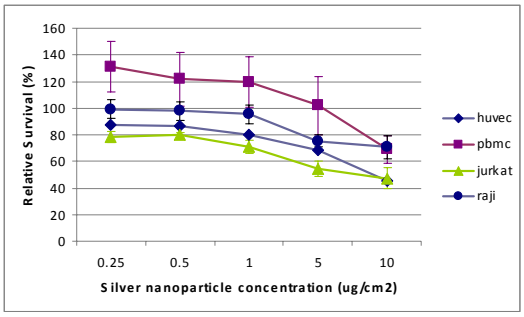

Figure 2. Cytoxicity of 15 nm pegylated dialyzed silver nanoparticle following 24 h treatment. Relative survival of Pegylated AgNP treated cells was determined by the MTT assay. Results show averages of 3 experiments

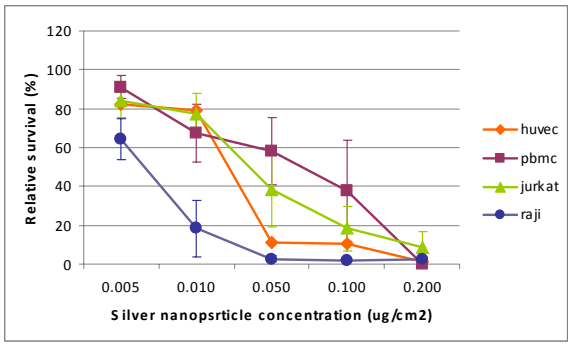

Figure 3. Cytoxicity of silver nanorod in human cells following 24 h treatment. Relative survival of cells was determined by the MTT assay. Results are the averages of 3 experiments.

3.2 Genotoxicity of 15 nm Ag NPs

Results of the cytokinesis-block micronucleus (CBMN) assays demonstrate that human peripheral blood cells treated with 0.32, 1.6, 3.2, 16, and 32 ug/ml Ag NPs for 72 h exhibited 26.5, 58.5, 53, 64, and 44 micronuclei (MNi; a biomarker of chromosome breakage or loss) per 1000 BN cells, respectively (Figure 4). Except for one treatment at the lowest Ag NP concentration all the results are significantly higher than that observed in the normal untreated control culture, normally in the range of 0–30 micronuclei (MNi). The Nuclear Division Index (NDI; an indicator of cell viability and proliferation) of these Ag NP-treated blood cells were determined to be 1.52, 1.27, 1.27, 1.2, 1.1 respectively (Figure 4), with most values lower than the scores (1.3 to 2.2) found in normal peripheral human blood cells. In addition, there were 2, 2, 3.3, 3.0, and 8.3 nucleoplasmic bridges (NPB; a biomarker of DNA misrepair/telomere end-fusion), respectively (data not shown).

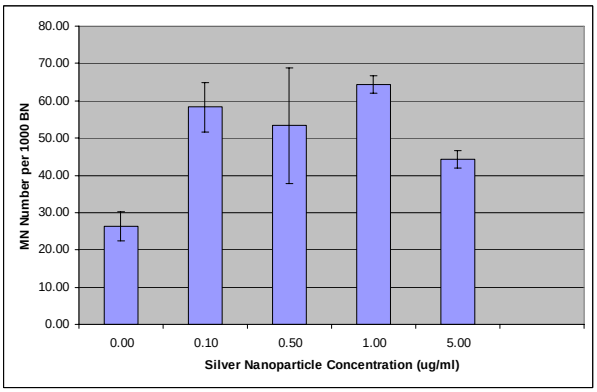

Figure 4. Genotoxicity of 15 nm Ag NPs in human peripheral blood following 72 h treatment. Micronuclei (MNi) formation in human blood cells was determined by the CBMN assay. Results are the averages of 3 experiments.

4. CONCLUSION

The results of MTT assays demonstrate that all the 3 types of engineered silver nanoimaterials tested, Ag NPs (15nm), PEGylated Ag NPs and silver nanorods, show various cytotoxic effects dependent on their physicochemical properties, treatment concentrations and duration as well as target cell types. The observation is in agreement with previous toxicity studies [e.g. 9,10]. Our studies using the CBMN assay demonstrate that Ag NP (15 nm) can also exert genotoxic effect in human blood cells. It will be of interest to investigate further the chromosomal damage patterns that could result in cells following exposure to silver nanomaterials. In view of the tremendous potential of silver nonomaterials in nanotechnology, our findings point to the urgent need for intensive and systemic investigation, both in vitro and in vivo, of the potential health and environmental impacts of these engineered products.

5. REFERENCES

[1] Balbus JM, et al. Environ Health Perpesct 115: 1664-1659, 2007
[2] Maynard AD. Ann. Occup. Hyg 51:1-12, 2007
[3] Stern TS, McNeil. Toxicol Sci 101:4-21, 2008
[4] Sharma VK, Yngard RA, Lin Y. Adv Colloid Interface Sc. 145:83-96, 2009
[5] Chen X, Schluesener HJ. Toxicol Lett. 176:1-12, 2007
[6] Fenech, M.. Nature Prot. 2:1084-1104 2007.
[7] Frens G. Nature 241:20-2, 1973.
[8] Otsuka H. Nagasaki Y, Kataoka K. Adv. Drug Delivery Rev.55: 403–419, 2003.
[9] Asharani PV, Wu YL, Gong Z, Valiyaveettil S. Nanotechnology 19 (2008) 255102 (8pp)
[10] Braydich-Stolle A, Hussain S, Schlager JJ, Hofman M-C. Toxicol Sci 88:412-419, 2005