Mutation Research 745 (2012) 4–10

Contents lists available at SciVerse ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Genotoxicity of silver nanoparticles evaluated using the Ames test and *in vitro* micronucleus assay☆

Yan Li[a], David H. Chen[b], Jian Yan[a], Ying Chen[a], Roberta A. Mittelstaedt[a], Yongbin Zhang[c], Alexandru S. Biris[d], Robert H. Heflich[a], Tao Chen[a,*]

[a] *Division of Genetic and Molecular Toxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, USA*
[b] *Little Rock Central High School, Little Rock, AR 72202, USA*
[c] *Nanotechnology Core Facility, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, USA*
[d] *Nanotechnology Center, University of Arkansas at Little Rock, Little Rock, AR 72204, USA*

ARTICLE INFO

Article history:
Received 12 November 2011
Received in revised form
18 November 2011
Accepted 18 November 2011
Available online 26 November 2011

Keywords:
Silver nanoparticles
Ames test
In vitro micronucleus assay
Genotoxicity

ABSTRACT

Silver nanoparticles (AgNPs) have antimicrobial properties, which have contributed to their widespread use in consumer products. A current issue regarding nanomaterials is the extent to which existing genotoxicity assays are useful for evaluating the risks associated with their use. In this study, the genotoxicity of 5 nm AgNPs was assessed using two standard genotoxicity assays, the *Salmonella* reverse mutation assay (Ames test) and the *in vitro* micronucleus assay. Using the preincubation version of the Ames assay, *Salmonella* strains TA102, TA100, TA1537, TA98, and TA1535 were treated with 0.15–76.8 μg/plate of the AgNPs. Toxicity limited the doses that could be assayed to 2.4–38.4 μg/plate; no increases in mutant frequency over the vehicle control were found for the concentrations that could be assayed. Human lymphoblastoid TK6 cells were treated with 10–30 μg/ml AgNPs, and additional cells were treated with water and 0.73 gy X-rays as vehicle and positive controls. Micronucleus frequency was increased by the AgNP treatment in a dose-dependent manner. At a concentration of 30 μg/ml (with 45.4% relative population doubling), AgNPs induced a significant, 3.17-fold increase with a net increase of 1.60% in micronucleus frequency over the vehicle control, a weak positive response by our criteria. These results demonstrate that the 5 nm AgNP are genotoxic in TK6 cells. Also, the data suggest that the *in vitro* micronucleus assay may be more appropriate than the Ames test for evaluating the genotoxicity of the AgNPs.

Published by Elsevier B.V.

1. Introduction

Nanomaterials have dimensions ranging from 1 to 100 nm and occur both naturally and as a result of manmade processes [1]. Because of their size, nanomaterials can have drastically different properties than their corresponding bulk (normal/not nanoscale) materials. These altered features can impart very desirable properties, including being less expensive, harder, and generally more efficient in particular industrial applications. Due to their increased use, exposure to nanomaterials is also expected to increase. With this exposure, there is a risk that the specific properties of nanomaterials could cause them to have adverse biological effects, such as toxicity and genotoxicity, which are different from their bulk counterparts. For example, while the bulk counterparts may be inert, the surface properties of some nanomaterials may lead to the formation of reactive oxygen species, that in turn may produce cellular damage, DNA adducts, and genotoxicity [2].

Silver has long been used as an antibacterial, antifungal, and antiviral agent because, while being very toxic towards microorganisms, it is much less toxic to humans [3]. However, antimicrobial agents such as silver nitrate are easily inactivated by complexation and precipitation and thus have a limited usefulness [4]. Zerovalent silver nanoparticles (AgNPs) are considered a valuable alternative to ionic silver. AgNPs have antimicrobial activity towards a broad spectrum of Gram-negative and Gram-positive bacteria, fungi, and viruses [5–8]. Therefore, dressings coated with sputtered nanoscale silver have been used to reduce infections in burns [9,10], and AgNPs have been used as antimicrobials in air fresheners, water purifiers, food storage containers, and in coatings for clothing. Because of these myriad applications, AgNPs are presently the most used engineered nanomaterials.

☆ The views presented in this article do not necessarily reflect those of the U.S. Food and Drug Administration.

* Corresponding author at: HFT 130, NCTR, 3900 NCTR Rd., Jefferson, AR 72079, USA. Tel.: +1 870 543 7954; fax: +1 870 543 7682.
E-mail address: tao.chen@fda.hhs.gov (T. Chen).

1383-5718/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.mrgentox.2011.11.010

The differences between the properties of nanomaterials and bulk materials generate uncertainty for measuring the genotoxic potential of nanomaterials using current genotoxicity assays. There is only a limited amount of data relating to the toxicity and genotoxicity of nanomaterials and, in some cases, the data are inconclusive or outright contradictory. From a regulatory standpoint, it is important to determine if the current genotoxicity assays used to evaluate the safety of new products are adequate for detecting the potential toxicity and/or genotoxicity of nanomaterials. The *Salmonella* reverse mutation assay (the Ames test) and the *in vitro* micronucleus assay are widely used genotoxicity assays and are included in many of the current batteries used for assessing genotoxic hazard [11]. One retrospective analysis suggested that a core *in vitro* genotoxicity battery comprising the Ames test plus the *in vitro* micronucleus assay was sufficient to detect rodent carcinogens and *in vivo* genotoxins [12].

The Ames test utilizes several different tester strains of *Salmonella typhimurium* to measure two classes of gene mutation, base pair substitution and small frameshifts [13,14]. Six of the eight Ames tests on nanomaterials thus far reported in the literature were negative. Titanium dioxide (TiO_2) nanoparticles [15] and zinc oxide nanoparticles [16], with or without UV light irradiation, were negative in *S. typhimurium* (and *Escherichia coli*) tester strains. Also, TiO_2 nanoparticles [17] and silica-overcoated magnetic nanoparticles labeled with rhodamine B isothiocyanate [18] tested negative, with or without an S9 metabolic activation. Similar negative results were reported for single-walled carbon nanotubes (SWCNT) in nonstandard *Salmonella* strains YG1024/YG1029 [19] and for a mixture of C_{60} and C_{70} fullerite in *S. typhimurium* (and *E. coli*) tester strains [20]. The only positive responses thus far reported were with water-soluble FePt nanoparticles capped with $(CH_3)_4NH_4OH$, which induced a weak positive response in TA100 [21], and fullerene C_{60} dissolved in polyvinylpyrrolidone, which was photo-mutagenic in strain YG3003 [22]. The mutagenicity of AgNPs has not yet been evaluated in this mutagenicity system.

The *in vitro* micronucleus assay detects small membrane bound DNA fragments (micronuclei) in the cytoplasm of interphase cells. The assay measures the clastogenicity (chromosome breakage) and aneugenicity (changes in chromosome number) of test chemicals in cells that have undergone cell division during or after exposure [23]. There are a number of studies on the genotoxicity of nanomaterials in the assay, with most of the positive responses involving tests conducted on TiO_2 nanoparticles [24–28]. Other nanoparticles such as a nano-sized cobalt–chromium alloy [29], SiO_2 [30] and water soluble C_{60} fullerenes ($C_{60}(OH)_{24}$) [31] also induced elevated micronucleus frequencies. While single-walled carbon nanotubes produced no significant increase in micronucleus frequency [19], multi-walled carbon nanotubes were positive in the assay [32]. In addition, starch-coated AgNPs tested positive in normal human lung fibroblast cells (IMR-90) and human glioblastoma cells (U251) using the cytokinesis blocked version of the micronucleus assay [33].

The purpose of this study was to determine whether or not 5 nm AgNPs can be tested successfully for genotoxicity hazard using the Ames test and the *in vitro* micronucleus test in TK6 human lymphoblastoid cells. Although bacterial mutagenicity assays are common to all genotoxicity batteries, the antibacterial properties of AgNPs may limit the concentrations of the test article that can be assayed. It is possible that the test may be inappropriate for evaluating AgNPs. Also, although AgNPs have tested positive for micronucleus induction in other systems, TK6 cells have recently been proposed as more appropriate than the rodent cell lines that are more commonly used for genotoxicity testing [34,35]. It is of interest, therefore, to evaluate the ability of AgNPs to induce micronuclei in TK6 cells.

2. Materials and methods

2.1. Characterization of silver nanoparticles

The size of the AgNPs (Novacentrix, Austin, TX, USA) was determined using transmission electron microscopy (TEM). The AgNPs were homogeneously dispersed in water, and one drop of the suspension was deposited on a TEM grid, dried, and evacuated before analysis. Images were collected using a field emission JEM-2100F (JEOL, Tokyo, Japan) equipped with a CCD camera.

UV–visible spectroscopy was performed using a Lambda 45 UV/Vis spectrophotometer (PerkinElmer, Waltham, MA, USA). Nanostructure size and zeta potential were measured in water and cell culture medium using a Zetasizer (Malvern, Worcestershire, UK) as described in our previous study [36].

Briefly, the nanoparticle samples were measured after dilution of a AgNP stock solution to 50 μg/ml in water, phosphate buffered saline, or 0.22-μm filtered culture media. These dilutions were vortexed and sonicated for 5 min to provide a homogenous dispersion. For the size measurement, 1 ml of the diluted dispersion AgNPs was transferred to a 1 cm^2 cuvette for dynamic size measurement; for zeta potential measurement, a Malvern zeta potential cell was washed three times with ultrapure water followed by transferring 850 μL of diluted dispersion AgNPs to this cell to measure the zeta potential. The concentration of the samples and experimental methods were optimized to assure the quality of the data. Sixty nm NIST standard gold nanoparticles were used in the validation of the instrument. Both size and zeta potential were measured at least three times. The data were calculated as the average size or zeta potential of AgNPs.

2.2. Ames test

Standard preincubation assays [37], without S9 activation, were conducted following Organization for Economic Cooperation and Development (OECD) TG471 recommendations [38]. *S. typhimurium* tester strains TA98, TA100, TA1535, TA1537 and TA102 were obtained from BioReliance Corporation (Rockville, MD, USA) and used for the assay. Ten different concentrations of AgNPs (0.15, 0.3, 0.6, 1.2, 2.4, 4.8, 9.6, 19.2, 38.4, 76.8 μg/plate) were dispersed in 100 μL sterilized water by vortexing for 5 min and then sonicating for 5 min, and incubated with the tester strains in a total volume of 200 μL for 4 h at 37 °C with shaking at 80 rpm. The samples were then diluted with 2 ml of molten top agar and poured on minimum salts agar; three replicates were conducted for each dose group and three independent assays were performed for statistical analysis. The positive controls employed for the assay were 2-nitrofluorene (3 μg/plate) for TA98, nitrofurantoin (5 μg/plate) for TA100, sodium azide (1 μg/plate) for TA1535, ICR-191 (1 μg/plate) for TA1537, and mitomycin C (0.2 μg/plate) for TA102.

2.3. Micronucleus assay

2.3.1. Cell culture and treatments

The TK6 human lymphoblastoid cells and materials for cell culture were purchased from the American Type Culture Collection (Manassas, VA, USA). TK6 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. To initiate an assay, 3×10^5 cells were transferred to T-25 flasks containing 5 ml of growth medium and incubated overnight. The cells then were treated in triplicate with 50 μL volumes of water containing sufficient amounts of dispersed AgNPs to produce final concentrations of 10, 15, 20, 25, and 30 μg/ml. The dispersion procedure for the AgNPs was as same as that used for Ames assay. Cultures serving as the positive control were treated with 0.73 gy of X-rays using an RS-2000 Biological Irradiator (Rad Source Technologies, Suwanee, GA, USA), while vehicle controls were treated with 50 μL sterilized water only. The cells then were incubated, with gentle shaking, for 28 h so that the vehicle controls would go through 1.5–2.0 cell divisions. The cells in two extra cultures were counted at the beginning of the treatment period, and each experimental culture was counted following the treatment to insure that the vehicle control cultures had replicated adequately and to estimate the cytotoxicity of the treatments. Cell counts were made with a Z1 Particle Counter from Beckman Coulter (Brea, CA, USA).

2.3.2. Flow cytometry

The micronucleus analysis was performed essentially as described for the High Content Protocol 1 in the instruction manual for the *In Vitro* MicroFlow™ Kit (Litron Laboratories, Rochester, NY, USA). The volume of culture containing 5×10^5 cells was determined for the vehicle controls and this volume was withdrawn from each cell culture and placed into 15 ml centrifuge tubes. The cells were then processed and stained as described in the kit instruction manual. The samples were analyzed using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). The stopping gate was set at 10,000 healthy nuclei and threshold parameters were set as recommended in the instruction manual. The hypodiploid channel (P1 events) was used to estimate the induction of aneuploidy. Three nanoparticle-only control samples also were analyzed with the cytometer to determine if there was any interference with the micronucleus measurement due to the nanoparticles.

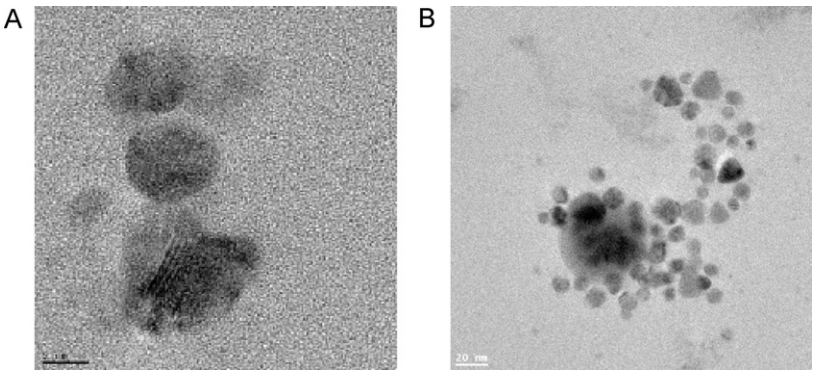

Fig. 1. Characterization of silver nanoparticles (AgNPs) by transmission electron microscopy (TEM). (A) Representative image of 5 nm AgNPs, was taken at 40,000×; (B) representative image of agglomeration of 5 nm AgNPs in water. Nanoparticles were deposited on formvar carbon coated grids and dried for TEM imaging. Images were analyzed in high resolution mode with an acceleration voltage of 100 kV.

2.3.3. Cytotoxicity analysis

Treatment-related cytotoxicity was estimated by relative population doubling (RPD) as recommended by OECD TG487 [23]. Information on cell death (apoptosis and necrosis) was gathered from the flow cytometer as described previously [39].

2.4. Data analysis

For the Ames test assay, positive responses required a dose-related increase in the number of revertant colonies/plate for one or more strains. Negative was defined as no dose-related increase in the number of revertant colonies. Also, a positive required that the number of revertant colonies/plate for at least one of the treatment concentrations was double the number of colonies/plate in the vehicle control. The maximum dose level tested for nontoxic test substances was 5 mg/plate.

For the micronucleus assay, three independent assays were conducted, each containing duplicate or triplicate treatments for each dose. Genotoxicity was expressed as the percent of micronuclei or hypodiploid events per 10,000 nuclei, and presented as the mean of 3 independent assays per dose ± SD. The data were analyzed for significance by one-way analysis of variance (ANOVA), followed by pairwise comparisons to the control using the Holm–Sidak method. A positive micronucleus response required that there was a concentration-dependent increase in micronucleus frequency and at least one treatment group induced a frequency that was significantly greater than that in the negative control. A response that had at least a 2-fold increase and 1% higher frequency relative to the vehicle control was considered a weak positive. A response that had at least a 3-fold increase and 3% higher frequency relative to the vehicle control was considered a strong positive result. Hypodiploid responses (P1 events) were confirmed similarly, with weak responses being at least 2-fold and 20/10,000 scored nuclei more than the vehicle control and strong responses at least 3-fold and 40/10,000 scored nuclei more than the vehicle control.

Statistical analysis was performed using SigmaPlot version 11.0 (SPSS, Chicago, IL, USA). All analyses were two-tailed, and $p < 0.05$ was used to identify statistically significant differences.

3. Results

3.1. Physical characterization of AgNPs

TEM analysis indicated that the AgNP test article had a size range of 4–12 nm (Fig. 1). Over 100 nanoparticles were measured and the distribution of the particle or aggregate sizes determined. 66% of particles measured had diameters in the range of 4 nm to 8 nm, while 24% of particles had diameters of 8–12 nm. In addition, 4% of AgNPs had diameters of 0–4 nm, and 6% of particles were above 12 nm in diameter. AgNP agglomeration occurred in the water diluted samples, producing agglomeration sizes up to 30 nm (Fig. 1). Also, UV–visible spectroscopy indicated that the AgNP sample had an absorbance maximum at about 450 nm, with a narrow peak width at half maximum, suggesting that the nanoparticles had a relatively narrow size distribution.

Zetasizer analysis indicated that the average size of AgNPs in ultrapure water was 61.2 ± 1.6 nm (mean ± SD), and the average size of the particles in culture medium was 1608.7 ± 175.4 (mean ± SD). The average surface charge of the AgNPs was −9.37 mV in water and −8.20 mV in media. TEM directly measured the primary size of the nanoparticles based on the projected area; while dynamic light scattering (DLS) measured the hydrodynamic diameter of the nanoparticles based on the translational diffusion area of the particle being measured. The same sample of AgNPs in water with a size of 61.2 nm by zetasizer analysis was measured as 30 nm agglomerates by TEM. This is due to the differences in the weighted averages determined by these two techniques, and also the differences in the physical properties measured. TEM is sensitive to the size of primary particles, whereas DLS is sensitive to the presence of small quantities of large particles or aggregates. The salt in media contributes to agglomeration potential and status and produces increases in agglomeration size.

3.2. Mutagenicity of AgNPs in the Ames test

Bacterial mutagenicity was assessed in *S. typhimurium* tester strains TA98 and TA1537 for detection of frameshift mutation and tester strains TA100, TA102, and TA1535 for measurement of base-pair substitution. Considering that the AgNPs were pure metal particles and unlikely to be metabolized by S9, the experiment was conducted only in the absence of S9. AgNP treatment of the different strains did not increase the mutant frequencies although the test article showed clear antibacterial ability (Table 1). The doses of AgNPs that were tested showed toxicity for all the tester strains, but individual strains exhibited different levels of sensitivity to the test article. For TA98 and TA100, frank toxicity (reduction of the background revertant frequency and/or thinning of the back-

Table 1
Mutagenicity of 5 nm silver nanoparticles in *Salmonella typhimurium* tester strains.

Dose (μg/plate)	Number of colonies/plate (mean ± SD)				
	TA1537	TA98	TA100	TA102	TA1535
0	11 ± 1	30 ± 4	153 ± 6	415 ± 6	20 ± 2
0.15	11 ± 2	26 ± 1	177 ± 6	387 ± 16	22 ± 3
0.3	12 ± 1	28 ± 5	161 ± 8	439 ± 21	14 ± 1
0.6	10 ± 2	29 ± 3	156 ± 14	448 ± 28	14 ± 2
1.2	12 ± 1	25 ± 4	144 ± 12	429 ± 21	10 ± 2
2.4	12 ± 4	23 ± 2	156 ± 10	415 ± 32	13 ± 2
4.8	13 ± 3	T	T	434 ± 14	10 ± 2
9.6	T			443 ± 16	T
19.2				429 ± 29	
38.4				485 ± 53	
76.8				T	
Positive control	644 ± 28*	758 ± 39*	788 ± 21*	1768 ± 43*	1050 ± 50*

Notes: Three independent assays were performed and SD represents standard deviation; T denotes toxicity detected at this and higher doses either as a reduction in the spontaneous frequency or a thinning of the background lawn.

* $p < 0.05$ vs. control.

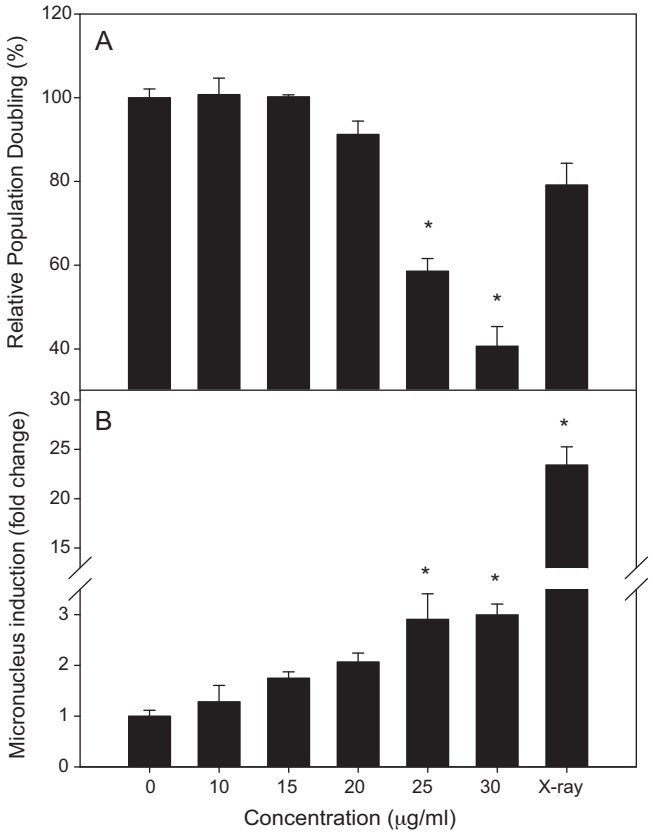

Fig. 2. Cytoxicity and genotoxicity of AgNPs in TK6 cells. (A) Cytotoxicity of 5 nm AgNPs in TK6 cells after 28 h treatment. Relative population doubling (RPD) was used to measure cell viability. (B) Induction of micronuclei by 5 nm AgNPs in TK6 cells. The micronucleus induction is expressed as fold change over the negative control (mean frequency, 0.53 ± 0.15%). X-rays were used as the positive control. The assays were performed in duplicate or triplicate in three independent experiments. $^{*}p < 0.05$ vs. control.

ground lawn) was detected at 4.8 μg/plate and higher; toxicity was apparent for TA1535 and TA1537 at 9.6 μg/plate and higher. At the highest dose of 76.8 μg/plate, all the bacteria from the 5 different strains were killed.

3.3. Micronucleus induction by AgNPs in TK6 cells

As part of the micronucleus assay, the cytotoxicity of AgNPs was evaluated in TK6 cells treated at concentrations of 10–30 μg/ml. As suggested by OECD TG487 [23], cytotoxicity measurements were made using RPD, which reflects a combination of cell growth, death, and cytostasis. The pooled cytotoxicity data from three independent assays conducted on AgNPs are displayed in Fig. 2A and show that AgNPs had a major increase in cytotoxicity above concentrations of 20 μg/ml. Treatment with 30 μg/ml AgNPs resulted in an RPD of 45.4%, just at the limit for maximum toxicity in the assay of 45 ± 5% RPD (55% ± 5% cytotoxicity).

Micronucleus frequencies were increased by the AgNP treatment in a dose-dependent manner (Fig. 2B), with significant increases in micronuclei measured at both 25 and 30 μg/ml. The 25 μg/ml treatment produced a 2.59-fold increase over the vehicle control with a net increase of 1.02% and 30 μg/ml produced a slightly greater response of 3.17-fold over the control with a net increase of 1.60%, both considered to be, weak positive responses by our criteria (see Section 2). The positive control X-ray treatment resulted in a 76.3% RPD and 20-fold induction in micronucleus frequency over the control. No micronuclei were detected when the AgNPs-only control samples were assayed, indicating that the

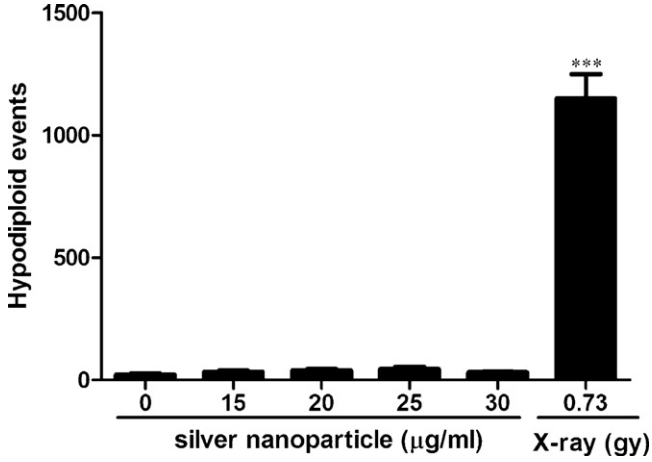

Fig. 3. Hypodiploid induction by AgNPs in TK6 cells. The effect on aneugenicity was shown as the frequency of hypodiploid events/10,000 intact nuclei. The assays were performed in duplicate or triplicate in three independent experiments. $^{***}p < 0.001$ vs. control.

nanoparticles and their agglomerates did not interfere with the measurement of micronuclei.

Hypodiploid events (representative of aneugenicity) also were scored using the flow cytometer and the results are shown in Fig. 3. No hypodiploid induction was found.

4. Discussion

AgNPs have been utilized extensively in health, electronic, and home products. Following their increasing use, more and more concerns have been raised about safety issues, including their potential genotoxicity. Herein, we applied two OECD standard assays, the Ames test and the *in vitro* micronucleus assay, to evaluate the suitability of current genotoxicity tests for assessing the genotoxicity of nanoparticles.

Our findings indicate that 5 nm AgNPs are negative in the Ames test (Table 1) and this result is consistent with previous reports indicating that nanoparticles, in general, are Ames negative [15,17,18,20,40,19] or weakly positive [22,41]. It has been suggested that the negative results may be due to the inability of the nanomaterials to penetrate the bacterial cell wall [42,43]. Insoluble particles generally enter cells via phagocytosis and the bacterial cell wall might not be able to perform this function so the nanoparticles cannot enter bacterial cells to damage DNA and induce mutations. It may be significant in this regard that both the nanoparticles that were weakly positive in the Ames test were soluble [22,41]. Also, the agglomeration of the nanoparticles could make the particles too large to transport through the pores in bacterial cell wall.

Another possibility is that the negative responses to nanoparticles in the Ames test are due to the insensitivity of most of the tester strains to oxidative DNA damage. For example, potassium bromate, a strong oxidizing agent, was negative in the Ames test with *S. typhimurium* strains TA98, TA1535, TA1537, and TA1588, and only weakly positive in TA100 at doses of 2–4 mg/plate [44,45]. In contrast, potassium bromate was strongly positive in the *Hprt* and mouse lymphoma *Tk* gene mutation assays [46,47]. Currently, oxidative stress is considered the primary mechanism by which nanomaterials elicit genotoxicity [43,48–51]. Previously we found that the same AgNP test article as used in this study induced mutations in the *Tk* gene of mouse lymphoma cells via the generation of oxidative DNA damage, as demonstrated by an analysis of mutational types, positive responses in the oxidative Comet assay, and the expression of genes related to oxidative stress [36]. Although, as recommended by OECD 471 [37], we included a tester strain that

is sensitive to oxidative DNA damage (TA102), none of the tester strains that we used were sensitive to the genotoxicity of AgNPs.

It is worthy of note that some oxidizing mutagens such as aldehydes, peroxides, and hydrogen peroxide are positive in Ames strains TA100 and TA102 [52,53]. Thus, it is possible that the type of oxidizing agents such as quinine, peroxide, hydroxyl radical, superoxide, singlet oxygen play an important role in the different responses of *Salmonella* strains to oxidative stress. Kim et al. suggest that oxidative stress produced by AgNPs could result from superoxide radical via activating membrane-bound NAD(P)H oxidase during AgNP uptake into cells [54]. The bacterial strains might not be sensitive to this type of oxidative species.

A further complication in assaying AgNPs for genotoxicity in the Ames test is the antimicrobial properties of the test article; in fact, these properties are the major reason for the widespread use of AgNPs in consumer products. This toxicity limited the test to concentrations of approximately 2–40 μg/plate, well below the 5000 μg/plate limit that is recommended for nontoxic test articles [34]. Thus, it is possible that the inherent toxicity of AgNPs to the test organisms may have reduced the sensitivity of the test. In general, it appears that a combination of the physical properties, toxicity, and mechanisms of genotoxicity of nanoparticles, in particular of AgNPs, may result in false negative results and an underestimation of their mutagenicity by the Ames test.

Concentration-dependent cytotoxicity was similarly observed in TK6 cells following exposure to AgNPs (Fig. 2A). However, the degree of cytotoxicity allowed testing concentrations up to an order of magnitude greater for micronucleus induction than could be assayed for gene mutation by the Ames test. The cytotoxicity produced in TK6 cells was similar to the toxicity produced by the same nanoparticles in a mouse lymphoma assay in our previous study [36] and with other studies performed in cultured mammalian cells with different types of AgNPs [55,56]. Previous genotoxicity studies have shown that AgNPs could induce DNA damage as measured by the Comet assay [57] and micronuclei as measured by the cytokinesis blocked micronucleus assay (CBMN) [33]. Various suggestions have been made regarding the mechanisms responsible for this possible cytotoxicity and genotoxicity, including reduced mitochondrial function, changes in cell morphology, oxidative stress, and/or disruptions in the cell cycle [33]. However, Park et al. [58] reported that 200 μg/ml AgNPs (mean diameter of 150 nm) displayed very low cytotoxicity with less than 12% apoptosis. This conflicting result may be due to the different sizes of AgNP used, i.e., the smaller the nanoparticle, the greater its toxicity [59]. No significant genotoxic responses were also reported in a mouse lymphoma assay (average 60 nm) [57] and an *in vivo* micronucleus assay (<100 nm) [60]. Based on these disparate observations, we conclude that additional effort should be made in testing the toxicity of a range of AgNP sizes in different genotoxic assays.

The doses of AgNPs tolerated by TK6 cells in our study were sufficient to demonstrate a dose-responsive increase in micronuclei, with a significant induction of micronuclei at a dose of 25 μg/ml (Fig. 2B). By our testing criteria, we consider the response to be a weak positive. It should be noted, however, that testing *in vitro* micronucleus induction by nanomaterials using flow cytometry has the potential for generation of artifacts if the NPs mimic either nuclei or micronuclei during the flow analysis. This does not appear to be a problem, at least for 5 nm AgNPs. Controls run with AgNPs only indicted that the routine gating strategy was sufficient to exclude the NPs from the analysis. Also manual scoring using the CBMN on AgNP-treated TK6 cells produced similar positive results (data not presented).

This general result of a weak positive response is consistent with findings from other studies testing various types of nanomaterials in mammalian cells. Treatment of human bronchial epithelial cells with 10 and 200 nm anatase-sized TiO_2 nanoparticles increased micronucleus frequencies 2.6- and 1.9-fold over the control [24]. Up to 72 h of exposure of Syrian hamster embryo fibroblasts to ultrafine TiO_2 resulted in about 1.5-fold induction in micronuclei [28]. Kang et al. found that nano-TiO_2 produced dose-dependent increases in micronucleus frequencies in peripheral blood lymphocytes of up to 2.5-fold [27]. Also, 100 and 200 μg/ml concentrations of starch-coated AgNPs increased both DNA damage, as measured by Comet assay, and cytokinesis blocked micronucleus frequencies in IMR-90 human lung fibroblasts and U251 human glioblastoma cells [33], with the increases in micronucleus frequencies in the order of 2-3-fold. The largest micronucleus induction was reported using the BNMN by Kawata et al. [61] who found a micronucleus frequency of $47.9 \pm 3.2\%$ in the AgNP 24 h treatment group compared with $2.1 \pm 0.4\%$ in the vehicle control. The different genotoxic responses may result partially from the different cell types and/or the AgNPs used in the study. The AgNPs (7–10 nm) used in the study were stabilized with polyethylenimine, and it is possible that the unique physico-chemical properties of these coated NPs may have resulted in the large differences in responses relative to assays conducted with uncoated AgNPs. Thus, based on the majority of data, it appears that various types of nanomaterials produce positive responses in the *in vitro* micronucleus assay, but responses are generally weak.

Previously we found that as little as 5 μg/ml of the same AgNPs used in the present report produced a 7-fold increase in mutant frequency in another standard *in vitro* mammalian cell assay, the mouse lymphoma gene mutation assay [36]. Thus, although the *in vitro* micronucleus assay in TK6 cells or the *in vitro* micronucleus assay in general, may be useful for assessing the genotoxicity of nanomaterials, the micronucleus assay may not be the most sensitive of the standard *in vitro* tests. The differences between this and previous studies could possibly be due to the different genotoxicity assays measuring different genotoxic endpoints and/or the different characteristics of the cell lines.

In conclusion, 5 nm AgNPs did not induce mutations in five different *S. typhimurium* strains recommended by OECD TG471. However, AgNPs displayed concentration-dependent cytotoxicity and genotoxicity in the human lymphoblast TK6 cell micronucleus assay. Although it was a weak response, AgNPs produced statistically significant increases in micronucleus frequency in the assay. The data suggest that the *in vitro* micronucleus assay may be more appropriate than the Ames test for evaluating the genotoxicity of the AgNPs. The results also suggest that future studies on developing a more suitable battery of assays for the assessment of nanoparticle genotoxicity should also consider particle size and coating as variables. Finally, additional mechanistic and cancer bioassay data on various nanoparticles will be necessary before the 'right' genotoxicity assay can be established.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

Y.L. is supported by an appointment to the Oak Ridge Institute for Science and Education. This project was partially funded by the CORES program from the U.S. Food and Drug Administration.

References

[1] C. Buzea, I.I. Pacheco, K. Robbie, Nanomaterials and nanoparticles: sources and toxicity, Biointerphases 2 (2007) MR17–MR71.
[2] C. Carlson, S.M. Hussain, A.M. Schrand, L.K. Braydich-Stolle, K.L. Hess, R.L. Jones, J.J. Schlager, Unique cellular interaction of silver nanoparticles: size-dependent generation of reactive oxygen species, J. Phys. Chem. B 112 (2008) 13608–13619.

[3] S. Silver, T. Phung le, G. Silver, Silver as biocides in burn and wound dressings and bacterial resistance to silver compounds, J. Ind. Microbiol. Biotechnol. 33 (2006) 627–634.
[4] B.S. Atiyeh, M. Costagliola, S.N. Hayek, S.A. Dibo, Effect of silver on burn wound infection control and healing: review of the literature, Burns 33 (2007) 139–148.
[5] S.W. Kim, K.S. Kim, K. Lamsal, Y.J. Kim, S.B. Kim, M. Jung, S.J. Sim, H.S. Kim, S.J. Chang, J.K. Kim, Y.S. Lee, An in vitro study of the antifungal effect of silver nanoparticles on oak wilt pathogen Raffaelea sp., J. Microbiol. Biotechnol. 19 (2009) 760–764.
[6] K.J. Kim, W.S. Sung, S.K. Moon, J.S. Choi, J.G. Kim, D.G. Lee, Antifungal effect of silver nanoparticles on dermatophytes, J. Microbiol. Biotechnol. 18 (2008) 1482–1484.
[7] J.S. Kim, E. Kuk, K.N. Yu, J.H. Kim, S.J. Park, H.J. Lee, S.H. Kim, Y.K. Park, Y.H. Park, C.Y. Hwang, Y.K. Kim, Y.S. Lee, D.H. Jeong, M.H. Cho, Antimicrobial effects of silver nanoparticles, Nanomedicine 3 (2007) 95–101.
[8] L. Lu, R.W. Sun, R. Chen, C.K. Hui, C.M. Ho, J.M. Luk, G.K. Lau, C.M. Che, Silver nanoparticles inhibit hepatitis B virus replication, Antivir. Ther. 13 (2008) 253–262.
[9] A. Agarwal, T.L. Weis, M.J. Schurr, N.G. Faith, C.J. Czuprynski, J.F. McAnulty, C.J. Murphy, N.L. Abbott, Surfaces modified with nanometer-thick silver-impregnated polymeric films that kill bacteria but support growth of mammalian cells, Biomaterials 31 (2010) 680–690.
[10] K. Madhumathi, P.T. Sudheesh Kumar, S. Abhilash, V. Sreeja, H. Tamura, K. Manzoor, S.V. Nair, R. Jayakumar, Development of novel chitin/nanosilver composite scaffolds for wound dressing applications, J. Mater. Sci. Mater. Med. 21 (2010) 807–813.
[11] M.C. Cimino, Comparative overview of current international strategies and guidelines for genetic toxicology testing for regulatory purposes, Environ. Mol. Mutagen. 47 (2006) 362–390.
[12] D. Kirkland, L. Reeve, D. Gatehouse, P. Vanparys, A core in vitro genotoxicity battery comprising the Ames test plus the in vitro micronucleus test is sufficient to detect rodent carcinogens and in vivo genotoxins, Mutat. Res. 721 (2011) 27–73.
[13] K. Mortelmans, E. Zeiger, The Ames Salmonella/microsome mutagenicity assay, Mutat. Res. 455 (2000) 29–60.
[14] B.N. Ames, F.D. Lee, W.E. Durston, An improved bacterial test system for the detection and classification of mutagens and carcinogens, Proc. Natl. Acad. Sci. U. S. A. 70 (1973) 782–786.
[15] Y. Nakagawa, S. Wakuri, K. Sakamoto, N. Tanaka, The photogenotoxicity of titanium dioxide particles, Mutat. Res. 394 (1997) 125–132.
[16] The Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers, Opinion Concerning Zinc Oxide (Colipa No. S 76), Adopted by the SCCNFP During the 24th Plenary Meeting of 24–25 June, 2003 (SCCNFP/0649/03), Available at http://ec.europa.eu/health/ph_risk/committees/sccp/documents/out222_en.pdf, 2003.
[17] D.B. Warheit, R.A. Hoke, C. Finlay, E.M. Donner, K.L. Reed, C.M. Sayes, Development of a base set of toxicity tests using ultrafine TiO_2 particles as a component of nanoparticle risk management, Toxicol. Lett. 171 (2007) 99–110.
[18] J.S. Kim, T.J. Yoon, K.N. Yu, B.G. Kim, S.J. Park, H.W. Kim, K.H. Lee, S.B. Park, J.K. Lee, M.H. Cho, Toxicity and tissue distribution of magnetic nanoparticles in mice, Toxicol. Sci. 89 (2006) 338–347.
[19] E.R. Kisin, A.R. Murray, M.J. Keane, X.C. Shi, D. Schwegler-Berry, O. Gorelik, S. Arepalli, V. Castranova, W.E. Wallace, V.E. Kagan, A.A. Shvedova, Single-walled carbon nanotubes: geno- and cytotoxic effects in lung fibroblast V79 cells, J. Toxicol. Environ. Health A 70 (2007) 2071–2079.
[20] T. Mori, H. Takada, S. Ito, K. Matsubayashi, N. Miwa, T. Sawaguchi, Preclinical studies on safety of fullerene upon acute oral administration and evaluation for no mutagenesis, Toxicology 225 (2006) 48–54.
[21] S. Maenosono, T. Suzuki, S. Saita, Mutagenicity of water-soluble FePt nanoparticles in Ames test, J. Toxicol. Sci. 32 (2007) 575–579.
[22] N. Sera, H. Tokiwa, N. Miyata, Mutagenicity of the fullerene C60-generated singlet oxygen dependent formation of lipid peroxides, Carcinogenesis 17 (1996) 2163–2169.
[23] OECD, In Vitro Mammalian Cell Micronucleus Test (Mnvit), OECD Guideline for Testing of Chemicals, No. 487, iccvam.niehs.nih.gov/SuppDocs/FedDocs/OECD/OECD-TG487.pdf, 2010.
[24] J.R. Gurr, A.S.S. Wang, C.H. Chen, K.Y. Jan, Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells, Toxicology 213 (2005) 66–73.
[25] Q. Rahman, M. Lohani, E. Dopp, H. Pemsel, L. Jonas, D.G. Weiss, D. Schiffmann, Evidence that ultrafine titanium dioxide induces micronuclei and apoptosis in Syrian hamster embryo fibroblasts, Environ. Health Perspect. 110 (2002) 797.
[26] J.J. Wang, B.J. Sanderson, H. Wang, Cyto- and genotoxicity of ultrafine TiO_2 particles in cultured human lymphoblastoid cells, Mutat. Res. 628 (2007) 99–106.
[27] S.J. Kang, B.M. Kim, Y.J. Lee, H.W. Chung, Titanium dioxide nanoparticles trigger p53-mediated damage response in peripheral blood lymphocytes, Environ. Mol. Mutagen. 49 (2008) 399–405.
[28] Q. Rahman, M. Lohani, E. Dopp, H. Pemsel, L. Jonas, D.G. Weiss, D. Schiffmann, Evidence that ultrafine titanium dioxide induces micronuclei and apoptosis in Syrian hamster embryo fibroblasts, Environ. Health Perspect. 110 (2002) 797–800.
[29] I. Papageorgiou, C. Brown, R. Schins, S. Singh, R. Newson, S. Davis, J. Fisher, E. Ingham, C.P. Case, The effect of nano- and micron-sized particles of cobalt–chromium alloy on human fibroblasts in vitro, Biomaterials 28 (2007) 2946–2958.
[30] J.J. Wang, B.J. Sanderson, H. Wang, Cytotoxicity and genotoxicity of ultrafine crystalline SiO_2 particulate in cultured human lymphoblastoid cells, Environ. Mol. Mutagen. 48 (2007) 151–157.
[31] Y. Niwa, N. Iwai, Genotoxicity in cell lines induced by chronic exposure to water-soluble fullerenes using micronucleus test, Environ. Health Prev. Med. 11 (2006) 292–297.
[32] J. Muller, I. Decordier, P.H. Hoet, N. Lombaert, L. Thomassen, F. Huaux, D. Lison, M. Kirsch-Volders, Clastogenic and aneugenic effects of multi-wall carbon nanotubes in epithelial cells, Carcinogenesis 29 (2008) 427–433.
[33] P.V. AshaRani, G. Low Kah Mun, M.P. Hande, S. Valiyaveettil, Cytotoxicity and genotoxicity of silver nanoparticles in human cells, ACS Nano 3 (2009) 279–290.
[34] A.M. Lynch, J.C. Sasaki, R. Elespuru, D. Jacobson-Kram, V. Thybaud, M. De Boeck, M.J. Aardema, J. Aubrecht, R.D. Benz, S.D. Dertinger, G.R. Douglas, P.A. White, P.A. Escobar, A. Fornace, M. Honma Jr., R.T. Naven, J.F. Rusling, R.H. Schiestl, R.M. Walmsley, E. Yamamura, J. van Benthem, J.H. Kim, New and emerging technologies for genetic toxicity testing, Environ. Mol. Mutagen. 52 (2011) 205–223.
[35] D. Kirkland, S. Pfuhler, D. Tweats, M. Aardema, R. Corvi, F. Darroudi, A. Elhajouji, H. Glatt, P. Hastwell, M. Hayashi, How to reduce false positive results when undertaking in vitro genotoxicity testing and thus avoid unnecessary follow-up animal tests: report of an ECVAM workshop, Mutat. Res./Genet. Toxicol. Environ. Mutagen. 628 (2007) 31–55.
[36] T. Chen, W. Ding, Y. Chen, X. Guo, Y. Zhang, A. Biris, P. Rice, S. Ali, A. Aidoo, M. Moore, N. Mei, Genotoxicity of nanosilver in mouse lymphoma cells, Toxicologist 108 (2010) 363.
[37] D.M. Maron, B.N. Ames, Revised methods for the Salmonella mutagenicity test, Mutat. Res. 113 (1983) 173–215.
[38] OECD, OECD Guideline for Testing of Chemicals: Bacterial Reverse Mutation Test, http://www.oecd.org/dataoecd/18/31/1948418.pdf, 1997.
[39] S.M. Bryce, J.C. Bemis, S.L. Avlasevich, S.D. Dertinger, In vitro micronucleus assay scored by flow cytometry provides a comprehensive evaluation of cytogenetic damage and cytotoxicity, Mutat. Res. 630 (2007) 78–91.
[40] E.K. Dufour, T. Kumaravel, G.J. Nohynek, D. Kirkland, H. Toutain, Clastogenicity, photo-clastogenicity or pseudo-photo-clastogenicity: genotoxic effects of zinc oxide in the dark, in pre-irradiated or simultaneously irradiated Chinese hamster ovary cells, Mutat. Res. 607 (2006) 215–224.
[41] S. Maenosono, R. Yoshida, S. Saita, Evaluation of genotoxicity of amine-terminated water-dispersible FePt nanoparticles in the Ames test and in vitro chromosomal aberration test, J. Toxicol. Sci. 34 (2009) 349–354.
[42] R. Landsiedel, M.D. Kapp, M. Schulz, K. Wiench, F. Oesch, Genotoxicity investigations on nanomaterials: methods, preparation and characterization of test material, potential artifacts and limitations—many questions, some answers, Mutat. Res. 681 (2009) 241–258.
[43] H. Nabeshi, T. Yoshikawa, K. Matsuyama, Y. Nakazato, S. Tochigi, S. Kondoh, T. Hirai, T. Akase, K. Nagano, Y. Abe, Y. Yoshioka, H. Kamada, N. Itoh, S. Tsunoda, Y. Tsutsumi, Amorphous nanosilica induce endocytosis-dependent ROS generation and DNA damage in human keratinocytes, Part Fibre Toxicol. 8 (2011) 1.
[44] Y. Kurokawa, A. Maekawa, M. Takahashi, Y. Hayashi, Toxicity and carcinogenicity of potassium bromate—a new renal carcinogen, Environ. Health Perspect. 87 (1990) 309–335.
[45] M. Ishidate, T. Sofuni Jr., K. Yoshikawa, M. Hayashi, T. Nohmi, M. Sawada, A. Matsuoka, Primary mutagenicity screening of food additives currently used in Japan, Food Chem. Toxicol. 22 (1984) 623–636.
[46] G. Speit, S. Haupter, P. Schutz, P. Kreis, Comparative evaluation of the genotoxic properties of potassium bromate and potassium superoxide in V79 Chinese hamster cells, Mutat. Res. 439 (1999) 213–221.
[47] K. Harrington-Brock, D.D. Collard, T. Chen, Bromate induces loss of heterozygosity in the thymidine kinase gene of L5178Y/Tk(+/−)-3.7.2C mouse lymphoma cells, Mutat. Res. 537 (2003) 21–28.
[48] E.J. Petersen, B.C. Nelson, Mechanisms and measurements of nanomaterial-induced oxidative damage to DNA, Anal. Bioanal. Chem. 398 (2010) 613–650.
[49] J.L. Kang, C. Moon, H.S. Lee, H.W. Lee, E.M. Park, H.S. Kim, V. Castranova, Comparison of the biological activity between ultrafine and fine titanium dioxide particles in RAW 264.7 cells associated with oxidative stress, J. Toxicol. Environ. Health A 71 (2008) 478–485.
[50] Y.H. Hsin, C.F. Chen, S. Huang, T.S. Shih, P.S. Lai, P.J. Chueh, The apoptotic effect of nanosilver is mediated by a ROS-and JNK-dependent mechanism involving the mitochondrial pathway in NIH3T3 cells, Toxicol. Lett. 179 (2008) 130–139.
[51] L.K. Limbach, P. Wick, P. Manser, N. Robert, A. Bruinink, W.J. Stark, Exposure of engineered nanoparticles to human lung epithelial cells: influence of chemical composition and catalytic activity on oxidative stress, Environ. Sci. Technol. 41 (2007) 4158–4163.
[52] D.E. Levin, M. Hollstein, M.F. Christman, E.A. Schwiers, B.N. Ames, A new Salmonella tester strain (TA102) with A × T base pairs at the site of mutation detects oxidative mutagens, Proc. Natl. Acad. Sci. U. S. A. 79 (1982) 7445–7449.
[53] D. Dillon, R. Combes, E. Zeiger, The effectiveness of Salmonella strains TA100, TA102 and TA104 for detecting mutagenicity of some aldehydes and peroxides, Mutagenesis 13 (1998) 19–26.

[54] H.R. Kim, M.J. Kim, S.Y. Lee, S.M. Oh, K.H. Chung, Genotoxic effects of silver nanoparticles stimulated by oxidative stress in human normal bronchial epithelial (BEAS-2B) cells, Mutat. Res. (2011).
[55] M. Ahamed, R. Posgai, T.J. Gorey, M. Nielsen, S.M. Hussain, J.J. Rowe, Silver nanoparticles induced heat shock protein 70, oxidative stress and apoptosis in *Drosophila melanogaster*, Toxicol. Appl. Pharmacol. 242 (2010) 263–269.
[56] M. Ahamed, M. Karns, M. Goodson, J. Rowe, S.M. Hussain, J.J. Schlager, Y. Hong, DNA damage response to different surface chemistry of silver nanoparticles in mammalian cells, Toxicol. Appl. Pharmacol. 233 (2008) 404–410.
[57] Y.J. Kim, S.I. Yang, J.C. Ryu, Cytotoxicity and genotoxicity of nano-silver in mammalian cell lines, Mol. Cell. Toxicol. 6 (2010) 119–125.
[58] S. Park, Y.K. Lee, M. Jung, K.H. Kim, N. Chung, E.K. Ahn, Y. Lim, K.H. Lee, Cellular toxicity of various inhalable metal nanoparticles on human alveolar epithelial cells, Inhal. Toxicol. 19 (2007) 59–65.
[59] V. Vishwakarma, S.S. Samal, N. Manoharan, Safety and risk associated with nanoparticles—a review, J. Miner. Mater. Charact. Eng. 9 (2010) 455–459.
[60] Y.S. Kim, J.S. Kim, H.S. Cho, D.S. Rha, J.M. Kim, J.D. Park, B.S. Choi, R. Lim, H.K. Chang, Y.H. Chung, I.H. Kwon, J. Jeong, B.S. Han, I.J. Yu, Twenty-eight-day oral toxicity, genotoxicity, and gender-related tissue distribution of silver nanoparticles in Sprague-Dawley rats, Inhal. Toxicol. 20 (2008) 575–583.
[61] K. Kawata, M. Osawa, S. Okabe, In vitro toxicity of silver nanoparticles at noncytotoxic doses to HepG2 human hepatoma cells, Environ. Sci. Technol. 43 (2009) 6046–6051.