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Factors affecting the *in vitro* micronucleus assay for evaluation of nanomaterials

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Abstract

A number of *in vitro* methodologies have been used to assess the genotoxicity of different nanomaterials, including titanium dioxide nanoparticles (TiO_2 NPs) and silver nanoparticles (AgNPs). The *in vitro* micronucleus assay is one of the most commonly used test methods for genotoxicity evaluation of nanomaterials. However, due to the novel features of nanomaterials, such as high adsorption capacity and fluorescence properties, there are unexpected interactions with experimental components and detection systems. In this study, we evaluate the interference by two nanoparticles, AgNPs and TiO_2 NPs, with the *in vitro* micronucleus assay system and possible confounding factors affecting cytotoxicity and genotoxicity assessment of the nanomaterials including cell lines with different *p*53 status, nanoparticle coatings and fluorescence, cytochalasin B, fetal bovine serum in cell treatment medium and different measurement methodologies for detecting micronuclei. Our results showed that micronucleus induction by AgNPs was similar when evaluated using flow cytometry or microscope, whereas the induction by TiO_2 NPs was different using the two methods due to TiO_2's fluorescence interference with the cytometry equipment. Cells with the mutated *p*53 gene were more sensitive to micronucleus induction by AgNPs than the *p*53 wild-type cells. The presence of serum during treatment increased the toxicity of AgNPs. The coatings of nanoparticles played an important role in the genotoxicity of AgNPs. These collective data highlight the importance of considering the unique properties of nanoparticles in assessing their genotoxicity using the *in vitro* micronucleus assay.

Introduction

Nanomaterials have been rapidly developed and increasingly applied in manufacturing industries and consumer products due to their unique physico-chemical properties. With increasing human exposure to products containing nanomaterials, hazard identification methods to evaluate the safety of these nanomaterials become essential. Given the advantages of *in vitro* assays such as lower cost, few associated ethical problems, larger numbers of replicates possible (1), a number of *in vitro* assays have been used to assess the toxicity of nanomaterials including cell viability, oxidative stress, inflammation and genotoxicity. However, the current *in vitro* assays are designed and validated for bulk materials. The suitability and adaption of these *in vitro* assays for the risk assessment of nanomaterials need further evaluation (2,3).

Nanomaterials possess unique features such as small size, surface charge, optical and magnetic properties and catalytic activity. These properties may result in interactions between the nanomaterials and experimental components. Carbon nanomaterials have been found to interfere with a number of cell viability

assays due to adsorption of neutral red or interaction with the tetrazolium salts in the 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) assay (4) and other cytotoxicity indicators, such as Commassie Blue and Alamar Blue™ (5,6). In addition, nanomaterials can interact with fluorescent dyes. Dextran-coated iron oxide nanoparticles (7), uncoated and oleic-acid coated Fe_3O_4 (4) and carbon nanoparticles (8) quenched the fluorescence responses and decreased the fluorescence emission of 2′,7′-dichlorofluorescin (DCF), oxidative form of 2′,7′-dichlorofluorescin-diacetate (DCFH-DA), a widely used fluorescence indicator for oxidative stress. While other nanoparticles increased the DCF fluorescence after exposure to the cells such as TiO_2 NPs, single-walled carbon nanotubes (SWCNTs), fullerene and silica particles (3). The fluorescence spectrum of acridine and amino coumarin derivatives was changed by polyvinylpyrrolidone (PVP)-coated silver nanoparticles in 2-propanol (9). Fluorescence quenching by nanomaterials might result from the binding of dyes onto the surface of the nanoparticles. Cytochalasin B was found to inhibit the endocytosis of TiO_2 NPs in the cytokinesis-blocked micronucleus assay, which caused a potential problem for appropriately measuring genotoxicity of the nanomaterial (3,10). All these reports suggested that the test results from some *in vitro* methodologies for nanomaterials can be misleading. Thus, study design and the relevant factors should be well considered for risk assessment of nanomaterials.

In this study, we examined the genotoxicity of two most widely used nanoparticles, silver nanoparticles (AgNPs) and titanium dioxide nanoparticles (TiO_2 NPs). The *in vitro* micronucleus assay, one of the more sensitive and frequently used mammalian cell assays for genotoxicity (11,12) was used for this evaluation. Different conditions were applied to determine the effect of experimental components and methodologies, cell types and unique characteristics of nanoparticles on the detection of micronucleus formation, as well as the cytotoxicity in the assay.

Materials and methods

Characterisation of AgNPs and TiO2 NPs

Five nanometer uncoated AgNPs obtained from Novacentrix (Austin, TX, USA), 5 nm PVP-coated AgNPs purchased from Nanocomposix (San Diego, CA, USA) and 10 nm uncoated TiO2 NPs synthesised as previously described (13) were used for this study. All of these nanomaterials have been well characterised and the data have been published previously (14–16).

Stock solution preparation of AgNPs and TiO2 NPs

Five nanometer uncoated AgNPs solution was vortexed and sonicated for 5 min in ultrasonic water bath (Branson 2510, Branson Ultrasonics, Danbury, CT, USA) to provide a homogenous dispersion. Five nanometer PVP-coated AgNPs were sonicated for 30 s in ultrasonic water bath according to the manufacture's instruction and TiO_2 NPs were dispersed in sterilised water by Vibra-Cell™ probe sonication (Sonics & Materials, Inc. Newtown, CT, USA) with 117 volts, 50/60 Hz for 10 min.

Particle size and zeta potential of the AgNPs and TiO_2 NPs in cell culture medium

To characterise the nanoparticles before and after treatment, 3 μg/ml 5 nm uncoated AgNPs, 1.5 μg/ml 5 nm PVP-coated AgNPs and 800 μg/ml TiO_2 NPs were suspended in 5 ml RPMI-1640 medium containing 10% inactivated horse serum for 0, 3 and 28 h, then were harvested for size distribution and zeta potential analysis. The average size distribution and zeta potential analysis were measured by Malvern Instruments Zetasizer Nano ZS Dynamic Light Scattering system (DLS) with disposable plastic cuvettes. All measurements were performed in triplicate at 25°C.

Cell culture

Human lymphoblastoid TK6 cells, L5178Y/Tk$^{+/-}$ –3.7.2C mouse lymphoma cells and Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). TK6 cells were maintained in RPMI-1640 medium (Life Technologies, Grand Island, NY, USA) and CHO cells were grown in F12 medium (Life Technologies). Both the media were supplemented with 10% fetal bovine serum (FBS) (Biologicals, Lawrenceville, GA, USA) and 1% penicillin/streptomycin (Life Technologies). L5178Y cells were cultured in Fischer's medium (Quality Biological, Gaithersburg, MD, USA), supplemented with 0.1% pluronic F68 (Life Technologies), 1 M sodium pyruvate (Life Technologies), 10% horse serum (Life Technologies), 100 U/ml penicillin and 100 μg/ml streptomycin. All the cells were maintained in a humidified incubator with 5% CO_2 in air at 37°C.

Cell treatment

To measure different cell line effects on the micronucleus assay, TK6, L5178Y and CHO cells were used. TK6 and L5178Y cells grow in suspension while CHO cells grow adherently. In view of the difficulty in separating the nanoparticles from the suspension cultures, TK6 and L5178Y were treated continually and the treatment time was determined according to the cell growth rate (1.5–2 doubling time). In contrast, CHO cells were treated for four hours and incubated in medium before counting the micronuclei. Prior to the treatment, 3×10^5 cells in 5 ml growth medium were seeded in T-25 flasks for TK6 cells and L5178Y cells or six-well plates for CHO cells for overnight incubation.

Treatment of 5 nm uncoated AgNPs in L5178Y cells.
A series of concentrations (0, 1, 1.5, 2, 2.5 and 3 μg/ml) of the 5 nm uncoated AgNPs were applied to treat L5178Y cells for 24 h continuously. Then the cells were harvested for the micronucleus assay by flow cytometry. 0.75 Gy of X-rays generated via an RS-2000 Biological Irradiator (Rad Source Technologies, Suwanee, GA, USA) was used as the positive control.

Treatment of 5 nm uncoated AgNPs in TK6 cells.
A series of concentrations (0, 10, 15, 20, 25 and 30 μg/ml) of the 5 nm uncoated AgNPs were applied to treat TK6 cells for 28 h continuously. Then the cells were harvested for the micronucleus assay by flow cytometry. 0.75 Gy of X-rays generated via an RS-2000 Biological Irradiator was used as the positive control.

Treatment of 5 nm PVP-coated AgNPs in TK6 cells.
Concentrations of 0, 1.5, 1.75 and 2 μg/ml 5 nm PVP-coated AgNPs in triplicates were used to treat TK6 cells for 28 h (two-doubling time) continuously in three independent experiments. Cell cultures were harvested and washed for the micronucleus assay analyzed by a flow cytometer, or washed and incubated for further 24 h with medium containing 3 μg/ml cytochalasin B for the micronucleus assay evaluated by microscopy, where 24-h treatment of 0.01 μg/ml Mitomycin C (MMC) was used as the positive control.

Treatment of 10 nm uncoated TiO_2 NPs in TK6 cells.
In the flow cytometry micronucleus assay, TK6 cells were treated with different concentrations of 0, 10, 50, 100, 200, 400 and 800 µg/ml TiO_2 NPs in duplicates for 28 h. In the microscope-based micronucleus test, TK6 cells were treated with 0, 100 and 200 µg/ml of TiO_2 NPs in triplicates for 28 h in three independent experiments. Four hour treatment of 0.125 µg/ml MMC was used as the positive control for both methods.

Treatment of 5 nm PVP-coated AgNPs in CHO cells.
CHO cells were treated with the 5 nm PVP-coated AgNPs (0, 1.5 and 1.75 µg/ml in triplicates) for 4 h in the treatment medium with or without 10% FBS in three independent experiments. Although uptake of the nanoparticles by cells was not displayed in this study, it was shown in other papers that AgNPs can enter into the mammalian cells (17,18). After 4-h treatment, cells were washed and incubated in cell growth medium for another 20 h. Then, the cells were collected for the micronucleus assay, analyzed using a flow cytometer.

Cytotoxicity determination
The estimated cytotoxicity of the nanomaterials was calculated based on cell counts which were made with both a Z1 Particle Counter from Beckman Coulter (Brea, CA, USA) and Trypan blue exclusion assay using Nikon TMS-F microscope (Nikon Corporation, Tokyo, Japan) at low magnification.

Micronucleus endpoint analyzed by flow cytometry
The micronucleus assay was performed using the *In Vitro* MicroFlow™ Kit (Litron Laboratories, Rochester, NY, USA). The whole procedure was conducted as described previously (16). The prepared 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. For the TiO_2 NPs study, 200 and 800 µg/ml TiO_2 NPs alone and control samples were also analyzed with the cytometer in three independent experiments to determine if there was any interference of the nanoparticles with the micronucleus measurement.

Binucleated micronuclei analyzed by microscopy
TK6 cells treated with the 5 nm PVP-coated AgNPs in duplicate cultures were prepared for the sequential cytokinesis-blocked micronucleus (CBMN) assay reported by Doak *et al.* (7). The cells were harvested as described previously (19). Briefly, after treatment, the cells were centrifuged and the pellet was suspended in 0.56% KCl and centrifuged immediately at 800 rpm for 10 min. The supernatant was discarded and the cells was put in fixative 1 solution [(methanol: acetic acid: 0.9% NaCl (5:1:6 parts)] and centrifuged again. The cells were then washed in fixative 2 solution [methanol: acetic acid (5:1 parts)] for four times and stayed in the fixative 2 solution overnight. Then, the cells were centrifuged and the pellets were resuspended to individualise the cells with approximately 500 µl of fixative 2. And 100–150 µl of the cells were pipetted slowly and evenly onto a slide wiped off with a dry cellulose cloth (best with one movement). The cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) for microscope based scoring. One slide containing 6000 cells per culture) has been analyzed using the Metafer Image Analysis System (MetaSystems, Carl Zeiss Ltd).

Mononucleated micronuclei analyzed by microscopy
TK6 cells treated with the 10 nm uncoated TiO_2 NPs were prepared for micronucleus assay as previously reported (20). Briefly, approximately 10^6 cells suspended in hypotonic 0.075 M KCl solution were incubated for 10 min at room temperature, fixed twice with ice-cold methanol containing 25% acetic acid, and then resuspended in methanol containing 1% acetic acid. A drop of the suspension was placed on a clean glass slide and air-dried. The cells were stained with 40 µg/ml acridine orange solution and immediately observed with a Carl Zeiss Axioskop fluorescence microscope (Carl Zeiss Ltd., UK). Three replicates were conducted for each treatment. At least 2000 intact interphase cells for each treatment were examined, and the cells containing micronuclei were scored.

Fluorescence spectra measurement of TiO_2 NPs
Fluorescence spectra of the TiO_2 NPs were measured by a Shimadzu 2550 Spectrophotometer (Shimadzu Corporation, Japan) using the quartz cuvette with a 1-cm optical path length in the range from 200 to 800 nm at room temperature. Fluorescence spectra were recorded with a fluorescence spectrophotometer from Photon Technology International (Edison, New Jersey, USA).

Statistical analysis
Statistical analysis was performed by one-way analysis of variance using SigmaPlot version 11.0 (SPSS, Chicago, IL, USA). All pairwise analyses were based on Holm–Sidak method and $P < 0.05$ was used to identify statistically significant differences.

Results

Characterisation of nanoparticles
The 5-nm uncoated AgNPs have primary size range of 4–12 nm, but agglomeration results in a secondary nanoparticle size of up to 30 nm in distilled water (14). The 5-nm PVP-coated AgNPs were well dispersed in water with less aggregation and obtained a similar average size of 2.6–5.7 nm in distilled water (15). The 10-nm uncoated anatase TiO_2 NPs had a primary size approximately of 8.9–15.3 nm, but were heavily aggregated in distilled water (the aggregated sizes was approximately 130–170 nm) (16).

After a 0, 3 and 28 h incubation of 3 µg/ml 5 nm uncoated AgNPs, 1.5 µg/ml 5 nm PVP-coated AgNPs and 800 µg/ml 10 nm uncoated TiO_2 NPs in culture medium containing 10% FBS, the size distribution analyses by Nanosight and DLS was shown in Table 3. Generally, agglomeration was observed for all the tested nanoparticles at all the time points. All the tested nanoparticle are stable through the end of treatment except more agglomeration occurred for 5 nm uncoated AgNPs with average size of 71.2 ± 2.12 nm when compared with the average size at 0 and 3 h after treatment (48.8 ± 0.65 and 47.7 ± 0.67 nm, respectively).

AgNPs responded differently in TK6 cells and L5178Y cells
The 5-nm uncoated AgNPs induced concentration-dependent cytotoxicity and genotoxicity in both TK6 cells (14) and L5178Y cells (Figure 1). However, L5178Y cells were more sensitive to the toxicity of AgNPs. The AgNPs reduced the growth rate to 50% relative population doubling (RPD) at 2.5 µg/ml in L5178Y, and at 30 µg/ml in TK6 cells (Figure 1A). At the concentrations causing the 50% RPD, the AgNPs induced similar fold changes of micronuclei over

their concurrent controls in both TK6 cells and L5178Y cells, indicating the AgNPs are more genotoxic to the mouse lymphoma cells than the TK6 cells because lower concentration of AgNPs were used for L5178Y cells (Figure 1B). In contrast, 0.75 Gy X-ray, the positive control, induced similar cytotoxicity and genotoxicity in both the cell lines (Figure 1).

Similar results for genotoxicity of AgNPs were obtained when micronuclei were measured using both flow cytometry and microscopy

There has been good agreement between the flow cytometry method for quantifying micronuclei and imaging based microscopy assessment for several clastogens and aneugens (21). However, no comparison study has been performed on nanoparticles. In this study, 1, 1.25 and 1.5 μg/ml of the AgNPs were used to treat TK6 cells for 28 h and micronucleus scoring was conducted using both the flow cytometry and microscopy methods (Figure 2). When comparing the micronucleus induction as fold increase over the concurrent negative control, similar results were obtained from the both methods. On the other hand, when the micronucleus formation was expressed as % micronucleus in 10 000 nuclei in flow cytometry method and % micronucleus in 2000 binuclei in microscopy examination, there is a slight difference between the two methods. As illustrated in Figure 2B, the micronucleus frequency resulting from microcopy-based scoring was significantly higher than that in the flow cytometry analysis method (Figure 2B).

Effects of FBS on the cytotoxicity and genotoxicity AgNPs

CHO cells were used for measuring effects of FBS because the cells can be treated for a short time and then separated from the particles to grow in medium with serum. The cells were treated with 1.5 and 1.75 μg/ml of the 5 nm PVP-coated AgNPs with or without 10% FBS for 4 h. Cytotoxicity was measured using RPD and genotoxicity was measured as fold change over the control. As shown in Figure 3, in the presence of 10% FBS, the AgNPs significantly induced cytotoxicity and genotoxicity in CHO cells. However, in the absence of the FBS, both cytotoxicity and genotoxicity were reversed completely to the control level (Figure 3).

TiO_2 NPs interfered with the particle counter and flow cytometer

According to the OECD TG487 (22), both RICC and RPD can be calculated based on the cell number of pre- and post-treatment in the absence of cytochalasin B (Cyto B) (see Table 1 note for formula). In this study, all the cell numbers were first obtained using Z1 Particle Counter, and the RICC and RPD are shown in Table 1. For each treatment, duplicate cultures were used. Up to 800 μg/ml, the data did not indicate any cytotoxicity. The cell growth with the TiO_2 NPs treatment seemed very close to the negative control cells. The 0.75 Gy X-ray treatments showed an average of 63.5 and 73.5% for RICC and RPD, respectively, which is consistent with our historical data for the cytotoxicity of X-ray (Table 1).

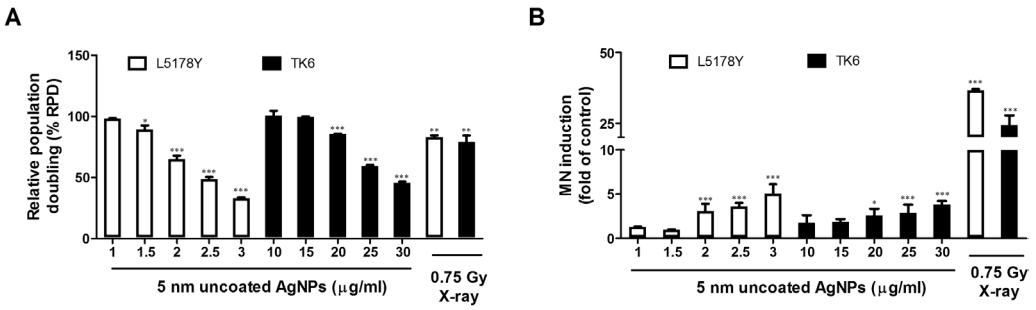

Figure 1. Effects of cell type on cytotoxicity and micronucleus induction by 5 nm uncoated AgNPs in L5178Y and TK6 cells. (**A**) Cytotoxicity of the AgNPs in L5178Y and TK6 cells. (**B**) Micronuclei induction by the AgNPs in L5178Y and TK6 cells. Relative population doubling (RPD) was used to measure cell viability. Micronucleus induction was expressed as fold change over the concurrent negative control. 0.75 Gy X-ray was used as the positive control. All the values were expressed as mean ± SD. The assays were performed in triplicate in three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the control.

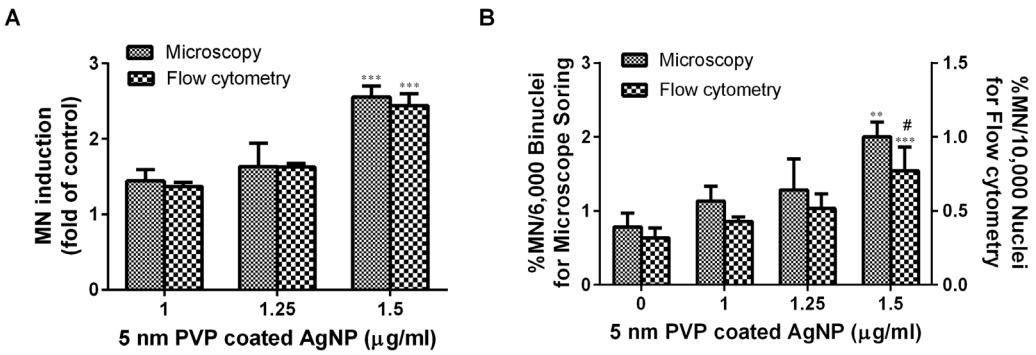

Figure 2. Comparison of micronuclei induction by 5 nm PVP-AgNPs in TK6 cells analyzed using microscopy and flow cytometry methods. (**A**) Micronuclei induction by the AgNPs in TK6 cells expressed as the fold change over the concurrent negative control. (**B**) Micronuclei induction by the AgNPs in TK6 cells expressed as percentage of micronuclei induction by counting 2000 binucleus cells using microscopy method and 10 000 mononucleus cells using flow cytometry method. All the values were expressed as mean ± SD. The assays were performed in triplicate in three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control; $^{\#}P < 0.05$ versus micronucleus frequency analyzed by microscopy method.

The flow cytometry micronucleus assay could also provide comprehensive information about cytotoxicity (21). As collected from the flow cytometer, 200 μg/ml of TiO_2 NPs induced an average of 52 % cytotoxicity, as calculated by the Relative Nuclei to Beads Ratio (RNBR). This was, however, in conflict with the cytostasis data generated by the RICC and RPD calculation based on automated cell counts. Moreover, the increased ratio of apoptotic and necrotic cells from 200 to 800 μg/ml further demonstrates the toxicity of TiO_2 NPs. In contrast, no significant induction of micronuclei was detected and in several exposure situations, the level of micronuclei present was lower than the control, suggesting errors in the measurement. At the same time, the fold change of micronucleus induction by 0.75 Gy X-ray was as expected and was greater than an 11-fold change over the concurrent negative control. This was in line with our historical data indicating the assay performed normally with the flow cytometer when a genotoxic, non-particulate material was used (Table 1).

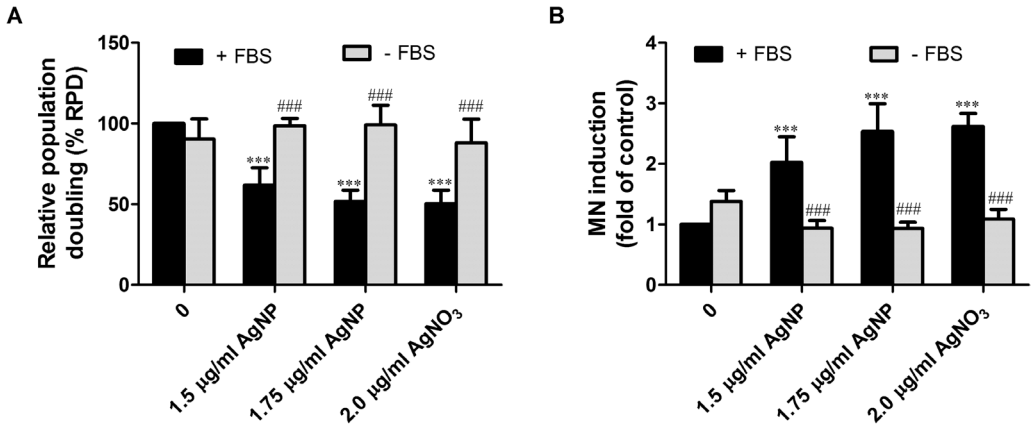

Figure 3. Effects of fetal bovine serum (FBS) on cytotoxicity and micronucleus induction by 5 nm PVP-coated AgNPs in CHO cells. (**A**) Cytotoxicity of the AgNPs in CHO cells with or without 10% FBS in cell treatment medium. (**B**) Micronucleus induction by the AgNPs in CHO cells with or without 10% FBS in cell treatment medium. Relative population doubling (RPD) was used to measure cell viability. Micronucleus induction was expressed as fold change over the concurrent negative control. All the values were expressed as mean ± SD. The assays were performed in triplicate in three independent experiments. $^{***}P < 0.001$ versus control; $^{###}P < 0.001$ versus groups with FBS in their treatment media.

Table 1. Cytotoxicity of 10 nm TiO_2 NPs measured with a Z1 particle counter and genotoxicity of the TiO_2 NPs evaluated using the flow cytometer micronucleus assay

Concentration of TiO_2 NPs (μg/ml)	Cytotoxicity				%MN
	%RICC	%RPD	%RNBR	% apoptotic/necrotic	
0-replicate 1	100	100	100	2.6	0.24
0-replicate 2	100	100	100	2.0	0.24
10-replicate 1	91	94	116	2.1	0.12
10-replicate 2	88	93	91	2.4	0.20
50-replicate 1	102	101	110	2.4	0.05
50-replicate 2	97	98	73	2.8	0.05
100-replicate 1	101	100	92	2.0	0.00
100-replicate 2	109	105	48	3.4	0.06
200-replicate 1	112	106	59	5.9	0.00
200-replicate 2	107	103	45	5.9	0.00
400-replicate 1	115	108	49	4.3	0.01
400-replicate 2	113	107	41	4.8	0.01
800-replicate 1	128	114	6	3.4	0.04
800-replicate 2	126	113	23	8.2	0.00
0.75 Gy X-ray-1	61	74	68	4.7	3.24
0.75 Gy X-ray-2	60	73	76	2.7	2.62

MN, micronucleus; TiO_2 NPs, titanium dioxide nanoparticles; RICC, relative cell count; RPD, relative population doubling.

RNBR, relative nuclei to bead ratio, an index stands for relative survival values. By scoring the 'Counting Beads' on the flow cytometer, the number of healthy nuclei per bead can be determined.

$$\text{RICC} = \frac{(\text{Increase in number of cells in treated cultures (final} - \text{starting))}}{(\text{Increase in number of cells in control cultures (final} - \text{starting))}} \times 100$$

$$\text{RPD} = \frac{\text{No. of population doublings in treated cultures}}{\text{No. of population doublings in control cultures}} \times 100$$

where population doubling = [log (Post-treatment cell number ÷ Initial cell number)] ÷ log 2.

After observing the abnormal readings from the cell counter and flow cytometer, we turned to measure the cytotoxicity using the Trypan blue exclusion assay utilizing manual microscopy based assessment, coupled to scoring micronuclei using a fluorescence microscope. The manual methods resulted in a substantially different data set (Table 2). The treatment of TiO_2 NPs at concentration of 200 μg/ml induced on average 54% RPD, which was similar to the 52% cytotoxicity determined by the flow cytometer. The treatment with 100 and 200 μg/ml TiO_2 NPs also resulted in a significant induction of micronuclei (2.73 ± 0.2 and 3.62 ± 0.4% fold-changes over the control, respectively).

To explore whether TiO_2 NPs could interfere the fluorescence counting of the instruments, we measured excitation and emission of fluorescence of TiO_2 NPs (Figure 4). It appears that TiO_2 NPs can emit fluorescence around 430 nm and the emission is dose-dependent. The emission could interfere micronucleus counting by the cytometry when fluorescence frequency was set at 488 nm for detecting fluorescent blue lights emitted by the antibodies attached to micronuclei.

Discussion

With increasing applications of nanomaterial-containing products, the need for knowledge on the potential hazards of nanomaterials is rising. Most of the studies have employed assays that have been designed to measure bulk materials to assess cytotoxicity and genotoxicity of nanomaterials. However, these assays designed for bulk chemicals are questionable for their capability for test of nanomaterials. For example, carbon nanomaterials may interfere with assay components or detection systems, such as ELISA assay and generate conflicting results (5,23–25). The Ames test, one of the commonly used genotoxicity assays, is not recommended for detecting the mutagenicity of nanomaterials due to the reduced ability of the insoluble nanoparticles to enter the prokaryotic cells (3,18). The interference of nanomaterials with micronucleus genotoxicity assays needs considering (26). In this study, we provide possible factors that can affect performance of the *in vitro* micronucleus assays using AgNPs and TiO_2 NPs as model nanomaterials.

Cells with different status of the p53 gene

The TK6 and mouse lymphoma L5178Y cell lines have been widely used for genotoxicity studies (27–30) and showed no significant differences when applying with *in vitro* micronucleus assay (31,32). However, L5178Y cells have a mutated *p53* ($p53^{+/-}$) tumor suppressor gene (33) while TK6 cells contain an intact *p53* ($p53^{+/+}$) gene (34).

Table 2. Cytotoxicity of 10 nm TiO_2 NPs measured with the trypan blue exclusive assay and genotoxicity of the TiO_2 NPs measured with the microscope micronucleus assay

Concentration (μg/ml)	Cytotoxicity		% MN
	RICC (%)	RPD (%)	
0	100 ± 0.0	100 ± 0.0	1.85 ± 0.3
100-TiO_2 NPs	73% ± 0.5***	77% ± 0.9***	2.73 ± 0.2***
200-TiO_2 NPs	58% ± 0.4***	54% ± 0.1***	3.62 ± 0.4**
0.125-MMC	70% ± 0.6***	71% ± 0.2***	5.07 ± 0.4***

MMC, mitomycin C; MN, micronucleus; TiO_2 NPs, titanium dioxide nanoparticles. **$p < 0.01$, ***$p < 0.001$ vs. control.

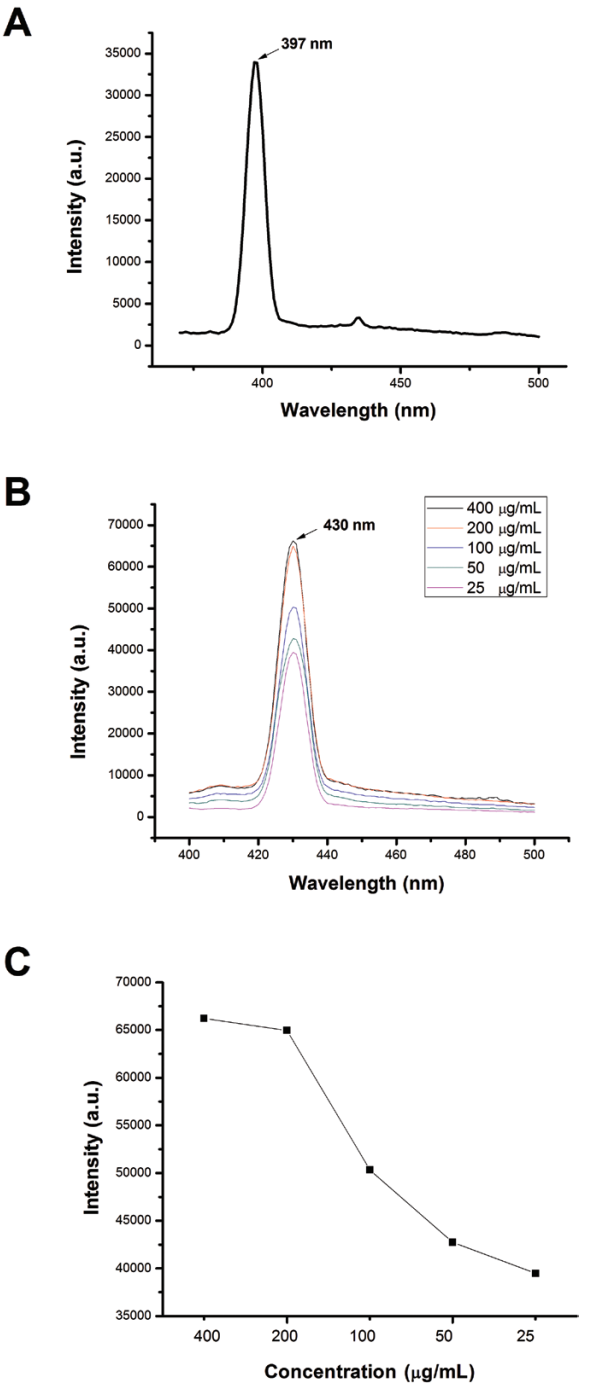

Figure 4. Fluorescence spectra of TiO_2 NPs. Fluorescence excitation (**A**) and emission (**B**) spectra of TiO_2 NPs in aqueous solution. The emission intensity is concentration-dependent (B and **C**).

Wild-type P53 protein functions as a regulator for cell cycle checkpoint and mediates apoptosis (35,36). Mutation in the tumor-suppressor *p53* gene frequently occurs in human and rodent tumors (37), and its loss or inactivation leads to chromosomal instability (38). Generally, model clastogens and spindle poisons such as X-rays and vincristine were more toxic to TK6 than to L5178Y cells because the p53 gene can initiate apoptosis of cells whose DNA is damaged by these mutagens (31). However, AgNPs caused more toxicity to L5178Y cells than to TK6 cells (Figure 1). In another study

Table 3. Particle size and zeta potential of AgNPs and TiO_2 NPs in the cell culture medium with different incubation time

Types of nanoparticles	Concentration (μg/ml)	Incubation time (h)	Z-average particle size (nm)	Zeta potential (mV)
5 nm uncoated AgNPs	3	0	49 ± 0.6	−9.97 ± 1.1
		3	48 ± 0.7	−9.78 ± 0.7
		28	71 ± 2.1	−10.8 ± 0.4
5 nm PVP-coated AgNPs	1.5	0	19 ± 0.2	−9.71 ± 0.4
		3	19 ± 0.3	−10.4 ± 1.3
		28	20 ± 0.2	−9.82 ± 0.5
10 nm uncoated TiO_2 NPs	800	0	868 ± 18	−9.85 ± 0.7
		3	892 ± 11	−10.3 ± 0.5
		28	860 ± 2.9	−9.95 ± 1.4

of ours, the similar results were found to other types of AgNPs with different sizes and coatings (39). Also, AshaRani *et al.* (40) found similar results in human glioblastoma cells (U251) where cells with mutated *p53* were more sensitive to micronucleus induction and DNA strand breaks induced by AgNPs than normal human lung fibroblast cells (IMR-90) with intact function of the *p53* gene. Thus, cell types should be well considered before they are used for evaluation of nanomaterials.

Methodology for *in vitro* micronucleus assay analysis

The cytokinesis-block micronucleus (CBMN) assay is a commonly used method in which cyto B, a cytokinesis inhibitor, is used to generate binucleated cells and these binucleated cells are scored using image analysis. Several studies have demonstrated the potential interference of cyto B on the prediction of nanomaterial genotoxicity (7,41). The flow cytometric micronucleus assay involves scoring the micronucleus frequency in mononuclear cells without cyto B and has been evaluated and validated for use with chemicals (42,43). It has generated equivalent results (42) to the cyto B method and is adopted by the OECD to evaluate micronucleus induction (22). In this study, we compared the two methods for evaluation of AgNP genotoxicity and found that there was no significant difference between the two methods (Figure 2). However, there was a large difference between the two methods for TiO_2 evaluation because the fluorescence from TiO_2 interfered with the flow scoring while the microscopy approach was not impacted. Hence, it is very important to understand the characterisation information of nanoparticles and select the most appropriate method for genotoxicity assessment.

Serum effects

There are several thousand different proteins in serum used in cell culture and these proteins can easily generate a protein corona around the surface of nanoparticles. There is a growing discussion on the interaction between nanoparticles and proteins (e.g. serum) and the effects of the protein corona on biological and toxicological functions of nanomaterials (44). The effects appear to be dependent on cell type and the unique properties of different nanomaterials (7), making it hard to reach a general conclusion about serum effects. Serum present in the culture medium reduced micronucleus induction by single-wall carbon nanotube in human lung epithelial cells while it increased the toxicity of amorphous silica nanoparticles to RAW 264.7 macrophage cells (45). In our study, an absence of FBS during exposure of CHO cells to 5 nm PVP-coated AgNPs reduced toxicity to the control level (Figure 3). The underlying mechanisms are still not clear. However, it is argued that small nanoparticles (<10 nm) can be actively bound to, solubilised by and carried by serum proteins, which facilitates nanomaterials presentation to the cells, resulting in their biological/toxicological effects (46). Our study along with others' highlights the effect of serum on the toxicological consequences of nanomaterials.

Interference with the cell counter and flow cytometer for the micronucleus assay

Cytotoxicity assessment was also included in parallel to our *in vitro* micronucleus assays. Normally cytotoxicity is determined by RPD calculated based on cell number or other cell viability assays using colorimetric dyes such as MTT or neutral red. Carbon-containing nanomaterials (5,23–25,47) and iron oxide nanoparticles (6,7) have been found to interact with the colorimetric dyes and generate misleading results. However, the interference with cytotoxicity assays was not limited to the colorimetric dyes. In this study, we found that TiO_2 NPs interacted with the cell counter and flow cytometer by affecting fluorescent dyes. It has been indicated previously that nanoparticles with heavy agglomerations can cause problem when using automatic cell counting instrument and deliver false cell counts information (3). The TiO_2 NPs used in this study fluoresced with an excitation/emission of 397/430 nm (Figure 4A and B) and the emission was concentration-dependent (Figure 4B and C). Failure to detect micronucleus induction by TiO_2 NPs using the flow assay may be due to the interference of TiO_2 NP agglomerations with fluorescent dyes used in micronucleus analysis by the flow cytometer via altering their emission intensity or quenching them altogether.

Coating materials

Nanomaterials have many unusual physicochemical properties, such as coatings, that may influence their toxicity (48). When AgNPs are coated with PVP, a hydrophilic material, it may limit the agglomeration and aggregation of AgNPs and stabilise the size distribution of AgNPs. On the other hand, uncoated AgNPs were easily aggregated and their size can increase from 5 to 30 nm in solution (14). Due to the coating effects, the 5-nm PVP-coated AgNPs (Figure 2) showed greater induction of toxicity than the 5-nm uncoated AgNPs (Figure 1) under the same treatment scheme. Similar impacts of coatings on the toxicity of silver nanomaterials also have been reported (49,50). A recent study focusing on comparing effects of different surface chemistry of quantum dots on their toxicity and genotoxicity shows that the surface chemistry has an influence on colloidal stability, agglomeration, cellular uptake and ion release, thus impacting cytotoxicity and genotoxicity of nanoparticles (51). Thus, these results suggest that coating of nanoparticles is a critical parameter impacting their cytotoxicity and genotoxicity. However, the silver ions released from 5 nm PVP-coated AgNPs were limited and did not play a major role in the genotoxicity of AgNPs (52).

In conclusion, there are several factors that can affect cytotoxicity and genotoxicity assessment of nanomaterials when measured using the *in vitro* micronucleus assay. These factors include the choice of cell lines with different *p53* status, nanoparticle coatings, fluorescence of nanomaterials, the use of cytochalasin B, fetal bovine serum in cell treatment medium and different measurement methodologies for *in vitro* micronucleus. Hence, our results highlight the importance to fully understand the physico-chemical features of nanomaterials under the experimental setting so that definitive associations between these parameters and any toxicological responses

observed can be identified. Also, our results suggest that choice of cell types, selection of methodology and modification of the existing methods should be considered carefully before genotoxicity evaluation of nanomaterials. We recommend using different conditions of the serum in medium for cell treatment to compare the impact of serum in the genotoxicity assay and microscopy micronucleus method for fluorescent NPs such as TiO_2 NPs.

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