Mutagenicity of ZnO nanoparticles in mammalian cells: Role of physicochemical transformations under the aging process

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Abstract

Zinc oxide nanoparticles (ZnO NPs) potentially undergo physicochemical transformation in the environment, which may lead to unexpected environmental and health risks. The “aging” process is essential for better understanding the toxicity and fate of NPs in the environment. However, the mutagenic effects of aged ZnO NPs are still unexplored. The present study focused on investigating the physicochemical transformation during aging process and clarifying the mutagenicity of naturally aged ZnO NPs in human–hamster hybrid (A½) cells. It was found that ZnO NPs underwent sophisticated physicochemical transformations with aging regardless of original morphology or size, such as the microstructural changes, the formation of hydrozincite (Zn₅(CO₃)₂(OH)₆) and smithsonite (ZnCO₃) and the release of free zinc ions. Interestingly, the aged ZnO NPs were investigated to be able to result in much lower cytotoxicity while relatively high degree mutation than fresh ZnO NPs. With characterization of the soluble and insoluble fractions of aged ZnO NPs suspension, together with the control measurements using metal chelator (TPEN) and endocytosis inhibitor (Nystatin), it was revealed that the release of zinc ions and nanoparticle uptake made significantly different contributions to the mutagenicity of fresh and aged ZnO NPs. This study clearly demonstrated that the physicochemical transformation of ZnO NPs with aging plays important and comprehensive roles in the ZnO NPs-induced mutagenicity in mammalian cells.

Introduction

Zinc oxide nanoparticles (ZnO NPs) have been widely applied in many different areas including cosmetics, coatings, sensors, packaging, biochemical imaging and catalysis, as well as some additives in foods and consumes (Li et al., 2011; Lv et al., 2012). With the increasing application and expanding production of ZnO NPs, the consequent release to the environment either by intended or accidental disposal is hardly avoidable. The modeled concentrations of ZnO NPs in natural surface water and treated wastewater in Europe have been estimated at 10 and 430 ng/L, respectively (Gottschalk et al., 2009). Due to the high surface to volume ratio and special reactivity, ZnO NPs inevitably undergo physicochemical transformation in the environmental media, potentially leading to unexpected environmental and health risks. However, the nature and extent of these transformations and the related toxicity properties are not fully understood yet.

Accumulating evidence has shown that ZnO NPs are inclined to dissolve and undergo structural transformations in response to aqueous environment (Lombi et al., 2012; Lv et al., 2012; Ma et al., 2013; Mudunkotuwa et al., 2012), in which the interaction interface is more active than that in the soil and atmospheric environment. ZnO NPs were reported to release much less Zn²⁺ in moderately hard water than in nanopure water (Poynton et al., 2011; Reed et al., 2012). Recent studies have shown that ZnO NPs were rapidly transformed to ZnS and Zn₃(PO₄)₂ during anaerobic digestion of wastewater and post-treatment processing of sewage sludge (Lombi et al., 2012; Ma et al., 2013). In a carbonate-rich water, hydrozincite (Zn₅(CO₃)₂(OH)₆) and smithsonite (ZnCO₃) were formed by precipitation of zinc ions released by the partial dissolution of ZnO NPs (Sivry et al., 2014). All these transformations have been expected to impact the bioavailability and toxicity of ZnO NPs in the aqueous system (Rathnayake et al., 2014). Aging, as physical or chemical transformations over time, is essential for understanding the fate of NPs in the environment (Lowry et al., 2012; Scheckel et al., 2010). However, related studies focusing on the aged ZnO NPs are limited. A comprehensive assessment on the kinetics of aging process to ZnO NPs transformation should be considered as an integral part of nanotoxicity investigation.

Among the documented toxic effects induced by ZnO NPs, the damage to DNA has been considered as a significant initiator of deleterious effects and plays a vital role in evaluating the potential health risk (Wright & Doak, 2009). A recent in vitro study revealed that ZnO NPs, independent of their size (≤35 nm or 50–80 nm), were able to increase significantly the frequency of micronuclei, indicating the generation of chromosome fragmentation or loss of partial chromosomes (Demir et al., 2014). Using the recently developed CometChip technology, Watson et al.
(2014) found a significant level of DNA damage due to ZnO NPs exposures in both suspension (TK6) and adherent (9H3T3) cells. These unrepair DNA damage may eventually result in point or deletion mutations. It was reported that ZnO NPs could induce marginal or weak mutagenic potential which might lead to frame-shift mutations in bacteria *Escherichia coli* WP2 trp uvrA and *Salmonella typhimurium* TA98 and TA1537 strains by bacterial reverse mutation assay (Ames test) (Kumar et al., 2011; Pan et al., 2010a). However, in mammalian cells, the mutagenicity of ZnO NPs remains largely unknown. Furthermore, since the prokaryotes lack the ability to perform endocytosis and ZnO NPs may be antibacterial (Emami-Karvani & Chehrazi, 2011), the mutation system based on the mammalian cells may be more relevant to environmental health. Therefore, our study reiterates the need to use mammalian cells as model for reevaluating environmental toxicity of ZnO presumably considered safe in environment.

Hence, to efficiently determine the mutagenic potential of ZnO NPs during aging, human–hamster hybrid (A<sub>L</sub>) cells containing a full set of hamster chromosomes and a single copy of human chromosome 11 were used in the present study. Since only a small segment of the human chromosome (11p15.5) is required for the viability of A<sub>L</sub> cells, mutations based on marker genes located in the human chromosome ranging in size up to 140 Mbp of DNA can be detected (Hei et al., 1998). This cell model is sensitive in detecting mutagens (such as asbestos fibers, particulate matters and certain heavy metals) which could induce mostly large, multilocus deletions (Bao et al., 2009; Xu et al., 2002). Here, we focused on the mutagenic property of ZnO NPs during the aging process and the underlying mechanism. In the meantime, TiO<sub>2</sub> NPs were included in the study for comparison as a metal oxide that is rarely dissolved in aqueous solution (Xia et al., 2008). Both ZnO NPs and TiO<sub>2</sub> NPs are most important inorganic UV filters that is rarely dissolved in aqueous solution (Xia et al., 2008). Both ZnO NPs and TiO<sub>2</sub> NPs are most important inorganic UV filters used in sunscreens (Burnett & Wang, 2011). Our data showed that ZnO NPs underwent dramatical physicochemical transformations with aging time ranging from 0 to 150 days. The pristine ZnO NPs in the dimension of 90–200 nm and 20 nm transformed into hydrozincite (Zn<sub>5</sub>(CO<sub>3</sub>)<sub>2</sub>(OH)<sub>6</sub>) accompanied by the release of Zn<sup>2+</sup> with the extension of aging time. We found that the aged ZnO NPs resulted in much lower cytotoxicity while relatively high degree mutation in A<sub>L</sub> cells. After separating the soluble and insoluble fractions of fresh and aged ZnO suspension by centrifugation, we confirmed that the mutation induction of aged ZnO NPs was closely related to the soluble fraction and partially mediated by the transformed particulates. Concurrent treatment with endocytosis inhibitor and metal chelator further showed that nanoparticle uptake and the release of zinc ions made significantly different contribution to the mutagenicity of fresh and aged ZnO NPs. With these results, we postulated that ZnO NPs combined with Zn<sup>2+</sup> contributed to the fresh NPs-inducing mutant fraction, while for aged ZnO NPs, the key contribution was resulted from more released Zn<sup>2+</sup> combined with newly-formed Zn<sub>5</sub>(CO<sub>3</sub>)<sub>2</sub>(OH)<sub>6</sub>. This study may help to better evaluate the comprehensive toxicity of ZnO NPs during the aging process in the environment.

**Methods**

**Nanoparticles and reagents**

ZnO NPs with an average primary particle size of either 90–200 nm (irregular morphology, 99.9% purity, referred to as ZnO 90–200 nm) or 20 nm (nearly spherical, 99.5% purity, referred as ZnO 20 nm) and anatase TiO<sub>2</sub> NPs with average size of 15 nm (spherical, 99.9% purity, referred as TiO<sub>2</sub> 15 nm) were purchased from Nanostructured & Amorphous Materials (Houston, TX). All chemicals, reagents and assay kits were purchased from Sigma-Aldrich (Shanghai, China).

**Preparation of aqueous dispersion of nanoparticles and exposure**

ZnO and TiO<sub>2</sub> nanopowder were suspended in Milli-Q water (Millipore, 18 MΩ cm) to 1 mg/mL concentration and sterilized by heating to 120 ºC for 30 min. The suspension was stored at room temperature for naturally aging period ranging from 0 day to 150 days. To ensure proper dispersion of nanoparticles, the stock solutions were vortexed and sonicated (30 W) before taking aliquots to prepare working solutions (in the medium F-12/8% fetal bovine serum (FBS)). Also, the working solutions were sonicated for 15 s at 30 W before they were dispersed into cell culture plates for further cellular assays.

**Physicochemical characterization of aqueous dispersion of nanoparticles**

All physicochemical characterizations listed below were carried out at room temperature. The hydrodynamic particle size of the sonicated nanoparticles was determined by dynamic light scattering (DLS), using a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK).

Transmission electron microscopy (TEM, JEOL 2100 HT, JEOL, Tokyo, Japan) was performed on fresh and aged ZnO or TiO<sub>2</sub> NPs at 100 µg/mL in Milli-Q water to evaluate the morphological changes of the NPs and determine if aggregation occurred as indicated by particle size analyses. The crystal phase and crystallinity were determined by energy dispersive X-ray spectrometer (EDX, JEOL 2100 HT, JEOL, Tokyo, Japan), high-resolution TEM (HRTEM, JEOL 2100 HT, JEOL, Tokyo, Japan) and X-ray diffraction (XRD, PANalytical B. V., Shanghai, China).

Inductively coupled plasma optical emission spectrometry (ICP-OES, Optima 7300 DV, Perkin Elmer Corporation, Norwalk, CT) was used to analyze the soluble fractions of fresh and aged NPs suspensions. After centrifugation at 20,000 rpm (28,000 g) for 1 h with cooling, the resulting zinc solution was applied for quantitative evaluation of elemental Zn (206,200 nm) and Ti (336,121 nm).

**Cell culture**

The human–hamster hybrid A<sub>L</sub> cells that contain a standard set of Chinese hamster ovary-K1 chromosomes and a single copy of human chromosome 11 were used in the present study. A<sub>L</sub> cells were cultured in Ham’s F12 medium (Gibco, Grand Island, NY) supplemented with 8% heated inactivated fetal bovine serum (Hyclone, Grand Island, NY), 2 × 10<sup>-4</sup> M glycine, and 25 µg/mL gentamicin at 37 ºC in a humidified 5% CO<sub>2</sub>/95% air incubator and passaged as previously described (Bao et al., 2009).

**Cytotoxicity and CD59 gene loci mutation assay**

Cells were seeded at a density of 8 × 10<sup>4</sup> cells 35-mm diameter Petri dishes in medium and incubated for 24 h. The medium was then replaced with 2 mL of medium containing various equivalent concentrations of fresh or aged NPs. The cells were incubated for 72 h. Then the treated cells were replated into 60mm diameter Petri dishes for cytotoxicity and CD59 gene loci mutation assay (Bao et al., 2009). The survival fraction was defined as the plating efficiency of treated group divided by the plating efficiency of the control group. The relative ratio of increase in mutation frequencies were expressed as fold increases over the background.

**Quantitative measurement of cellular uptake of nanoparticles by ICP-MS**

The inductively coupled plasma mass spectrometry (ICP-MS) was used to monitor the cellular Zn content after treatment with fresh and aged NPs. 5 × 10<sup>3</sup> A<sub>L</sub> cells treated with 50 µg/mL fresh or...
aged NPs for 2 h were collected and applied for quantitative measurement by ICP-MS Thermo X-Series II (Thermo Scientific, Bremen, Germany). Same number of cells without NPs treatment was used as an internal standard. Both standard and test solutions were measured for three times.

**Visualization of cell–particle interactions by TEM**

After treated with 50 μg/mL fresh and aged NPs for 2 h, the A549 cells were collected and fixed with 2.5% glutaraldehyde at 4 °C. After dehydration in ascending grades of ethanol, cells were subsequently embedded in epoxy resin. Ultrathin sections were performed using an ultramicrotome before observation using a Hitachi H7650 electron microscope (Beijing, China).

Newport Green™ DCF diacetate ester staining

NPs-treated A549 cells on glass slides were incubated for 1 h at 37 °C in the dark with Newport Green DCF diacetate ester at concentration of 2 μM, stained with Hoechst33342 nuclear stain (Beyotime, China) and finally visualized by confocal microscopy.

**Treatment with endocytosis inhibitor or metal chelator**

Nystatin (Sigma Aldrich, Shanghai, China), an endocytosis inhibitor, was diluted directly from stock solution with medium to a final concentration of 10 U/mL. TPEN (N,N,N',N'-tetakis(2-pyridylmethyl)ethylenediamine) as Zn2+ chelator was purchased from Sigma-Aldrich and dissolved in DMSO (dimethyl sulfoxide); the final concentration of TPEN for the inhibition assay was 3 μM. The final concentration of DMSO in culture did not exceed 1%. Nystatin or TPEN was added to the cell-incubation culture 30 min before the treatment coupled with NPs.

**Statistics**

Data from three to four independent experiments were subjected to statistical analysis. These data were represented as means ± SD. Data were analyzed with one-way analysis of variance (ANOVA). The results with p values of less than 0.05 were considered to be statistically significant.

**Results**

**Physicochemical transformations of ZnO NPs during aging**

To identify the microstructural changes with the aging process, fresh and the naturally aged NPs from 20 to 150 days were characterized. As shown in Figure 1(A), fresh ZnO 90–200 nm NPs were in irregular shape with rod/finger-like or nearly spherical and cubical crystals. After aged for more than 20 days, the microstructure of NPs was dramatically changed with the extension of aging time. The clear crystal morphology was fully disappeared when the aging time reached to 60 days. The surface of NPs aged for 60 days became amorphous and there were more sheet-like and needle particles, indicating that the dissolution process was gradually accelerated by the aging. When the pristine particle size of ZnO NPs was decreased to 20 nm, the anisotropic shape of fresh ZnO NPs turned into sheet-like NPs aged for 60 days as well. By contrast, TiO2 15 nm did not show any morphological transformation during the aging, except for more aggregation. Furthermore, the selected area electron diffraction (SAED) patterns confirmed that the crystal structure of ZnO 90–200 or 20 nm NPs changed from monocristalline to polycrystalline with the aging time (Figure 1A). The fresh ZnO 90–200 or 20 nm NPs exhibited a perfect crystal lattice with a clear HRTEM images, indicative of a highly crystalline structure. By contrast, the crystalline structures of 40-day aged ZnO 90–200 nm and 60-day aged ZnO 20 nm NPs turned to be poor and not uniform, which indicated that the crystallite sizes as well as the crystalline anisotropy were both deeply impacted by the aging time (Figure 1A). To determine the origin of the neoformation, aged ZnO NPs with 90–200 and 20 nm NPs were determined by EDX as shown in Figure S1. In the EDX spectra of aged ZnO 90–200 nm, the main constituent of Zn yielded the largest peak in both particles with clear boundaries and sheet-like particles. The similar EDX spectrum was observed in aged ZnO 20 nm. The XRD patterns of aged ZnO NPs finally confirmed the conversion of ZnO NPs to sheet-like hydrozincite (Zn5(CO3)2(OH)6) (Figure 1B).

Considering that ZnO NPs suspended in water converted to Zn5(CO3)2(OH)6 during aging, pH value would be expected to be different between fresh and aged stock suspensions. As shown in Table 1, there was an aging-time dependent enhancement of pH value in ZnO NPs stock suspensions. After aged for 60 days, pH value for ZnO 90–200 nm (or ZnO 20 nm) increased from 5.5 to 6.4 (or 6.8). When the aging time extended to 150 days, aged ZnO 90–200 nm suspension turned to be neutral (pH 7.2). In addition, the remarkable difference in hydrodynamic size between fresh and aged NPs, especially for ZnO 20 nm and TiO2 15 nm NPs were illustrated by DLS (Table 1). While after the solution was diluted into cell culture medium (F12/8% FBS), the hydrodynamic size of the agglomerates in the solution decreased significantly (Table 1).

To investigate the influences of natural aging process on dissolution of ZnO NPs prior to the exposure, the concentrations of soluble Zn supernatant of ZnO NPs dispersions in Milli-Q water at NPs concentrations of 50 μg/mL were tested by ICP-OES. As shown in Table 1, the concentrations of dissolved Zn in ZnO NPs suspension increased with aging, even though the increase was not so remarkable, with highest concentration in 150-day aged ZnO 90–200 nm NPs about 3.5 μg/mL. Our results showed that the dissolution of ZnO NPs could be enhanced to some extent by aging, accompanied with the transformation of the crystalline phase.

**Cytotoxicity of ZnO NPs decreased with aging time**

The viability of A549 cells exposed to graded doses of fresh and aged ZnO or TiO2 NPs was analyzed by colony formation assay. The normal plating efficiency of A549 cells was 86.55 ± 8.49. As shown in Figure 2(A), exposure A549 cells to graded doses of fresh ZnO 90–200 nm ranging from 1 to 15 μg/mL illustrated a dose dependent decrease of survival fractions. For fresh ZnO 90–200 nm, the lethal dose, which resulted in 100% cell killing, was 15 μg/mL. At a designated dosage of ZnO 90–200 nm, for example, 15 or 20 μg/mL, the survival fraction of exposed A549 cells showed an aging time-dependent increase tendency among the aged NPs-treated groups. The lethal dose of aged ZnO 90–200 nm was increased to 18.8, 32.5, 38.0 and above 60 μg/mL, when the aging time was 20, 40, 60 and 150 days, respectively. Likewise, the lethal dose for ZnO 20 nm was 15 μg/mL as well, while the survival fraction of 60-day aged ZnO 20 nm remained 49.06 ± 0.58 at the equal dose (Figure 2B). By comparison, aging time had minimal effect on the cytotoxicity of TiO2 15 nm. The survival fractions of both fresh and aged TiO2 at 15 μg/mL were above 70%.

**Mutation frequencies at CD59 gene loci in response to aged ZnO NPs**

To compare the mutagenic effects induced by different NPs, the relative ratio of increase in mutation frequencies were expressed as fold increases over the background. The average mutation
Figure 1. Physicochemical transformation of the crystalline phase of ZnO NPs and TiO₂ NPs with aging. (A) Low resolution TEM images with corresponding SAED (squares indicated) and HRTEM (circles indicated) images of fresh and aged NPs. (B) XRD patterns of Zn₅(CO₃)₂(OH)₆ reference, fresh and 60-day aged NPs.

Table 1. pH value, particle size and zinc concentration of fresh and aged NPs suspension.

| NPs        | Aging days | pH    | Hydrodynamic size (nm)/PdI | Zn Concentration (μg/mL) |
|------------|------------|-------|---------------------------|--------------------------|
|            |            |       | Milli-Q water | F-12/8% FBS | Milli-Q water | F-12/8% FBS |
| ZnO 90–200 nm | Fresh      | 5.5   | 921 ± 88/0.639 | 129 ± 44/0.657 | 2.5 ± 0.3 | 12.3 ± 0.3 |
|            | 20 days    | 5.8   | 1064 ± 80/0.701 | 127 ± 97/0.671 | 3.1 ± 0.2 | 14.0 ± 0.5 |
|            | 40 days    | 6.0   | 1323 ± 232/0.631 | 160 ± 42/0.605 | 3.1 ± 0.3 | 15.2 ± 0.3 |
|            | 60 days    | 6.4   | 1356 ± 203/0.648 | 177 ± 107/0.630 | 3.3 ± 0.4 | 15.6 ± 0.4 |
|            | 150 days   | 7.2   | 1709 ± 264/0.677 | 389 ± 185/0.639 | 3.5 ± 0.4 | 18.5 ± 0.6 |
| ZnO 20 nm  | Fresh      | 5.6   | 1977 ± 296/0.332 | 29 ± 1/0.672  | 2.6 ± 0.5 | 13.1 ± 0.2 |
|            | 60 days    | 6.8   | 4406 ± 1325/0.388 | 30 ± 4/0.688  | 3.6 ± 0.5 | 18.9 ± 0.6 |
| TiO₂ 15 nm | Fresh      | 5.5   | 950 ± 54/0.698  | 510 ± 32/0.444 | /            | /           |
|            | 60 days    | 5.5   | 2451 ± 256/0.701 | 706 ± 53/0.483 | /            | /           |

Nanoparticle size was expressed as intensity-based average hydrodynamic diameter. Bars: ±S.D. PdI = Polydispersity index. The dissolved Zn concentration was measured by ICP-OES: 50 μg/ml NPs was suspended in Milli-Q water at room temperature or in Ham’s F-12/8% FBS at 37°C for 72 h. The suspensions were centrifuged at 20000 rpm (28000 g) for 1 h, then the Zn concentrations in the supernatant were determined by ICP-OES.
background of A549 cells at CD59 gene locus used in the present experiments was 93 ± 24 mutants per 10⁵ survivors. As shown in Table 2, treatment of A549 cells with fresh ZnO 90–200 nm resulted in a dose-dependent induction of mutation yield at the CD59 gene locus. A significant increase in mutation yield over the background level was observed in cells exposed to fresh ZnO 90–200 nm at concentrations /C21 10 mg/mL (p < 0.05). With further increase of the concentration of fresh ZnO 90–200 nm to 15 mg/mL, no mutation was observed as a result that all treated cells were dead. The fold increases of mutation frequencies in cells exposed 20-, 40-, 60- and 150-day aged ZnO 90–200 nm reached a peak of 1.90 ± 0.21, 2.05 ± 0.20, 1.62 ± 0.13 and 1.63 ± 0.11, when the concentrations of aged NPs were 15, 20, 30 and 40 mg/mL, respectively. By comparison, the maximum fold increases of mutation frequencies for fresh TiO2 15 nm at a concentration of 10 mg/mL (p < 0.05) was 1.33 ± 0.11, which was roughly equivalent to 1.32 ± 0.05 that induced by 60-day aged TiO2 15 nm at a concentration of 15 mg/mL (p < 0.05). The data indicated that aging did not show obvious effect on TiO2 NPs-induced mutagenicity.

### Contribution of soluble and insoluble fractions to the mutagenicity of fresh and aged ZnO NPs

To evaluate the mechanism of toxicity of ZnO NPs with aging, the Zn element of cells exposed to fresh and aged ZnO NPs was first quantified by ICP-MS. As shown in Figure 3(A), the Zn amount (including both zinc ions and particles) of 60-day aged ZnO 90–200 nm and ZnO 20 nm was 38.735 ± 1.478 and 50.165 ± 2.185 ng, respectively, which was significantly higher than those of

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**Table 2. Mutagenicity of fresh and aged ZnO NPs and TiO2 NPs in A549 cells.**

| NPs                | Control | 1     | 5     | 10    | 12    | 15    | 20    | 30    | 40    | 50    |
|--------------------|---------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Fold increase of CD59⁻ mutation frequencies per 10⁵ survivors |
| ZnO 90–200 nm      |         |       |       |       |       |       |       |       |       |       |
| Fresh              | 1       | 0.85 ± 0.05 | 1.07 ± 0.08 | 1.26 ± 0.01 | 1.31 ± 0.07 | N.A.  | N.A.  | N.A.  | N.A.  | N.A.  |
| 20 days            | 1       | 1.10 ± 0.02 | 1.48 ± 0.19 | 1.55 ± 0.19 | 1.48 ± 0.16 | 1.90 ± 0.21 | N.A.  | N.A.  | N.A.  | N.A.  |
| 40 days            | 1       | 1.18 ± 0.09 | 1.32 ± 0.06 | 1.34 ± 0.03 | 1.61 ± 0.13 | 1.60 ± 0.08 | 2.05 ± 0.20 | N.A.  | N.A.  | N.A.  |
| 60 days            | 1       | 1.14 ± 0.07 | 1.33 ± 0.03 | 1.37 ± 0.06 | 1.40 ± 0.01 | 1.44 ± 0.13 | 1.62 ± 0.10 | 1.49 ± 0.06 | N.A.  | N.A.  |
| 150 days           | 1       | 1.11 ± 0.09 | 1.10 ± 0.07 | 1.19 ± 0.08 | 1.19 ± 0.07 | 1.25 ± 0.05 | 1.29 ± 0.16 | 1.48 ± 0.06 | 1.63 ± 0.11 | 1.44 ± 0.03 |
| ZnO 20 nm          |         |       |       |       |       |       |       |       |       |       |
| Fresh              | 1       | –     | 1.05 ± 0.06 | 1.48 ± 0.03 | –     | 1.29 ± 0.04 | –     | –     | –     | –     |
| 60 days            | 1       | –     | 1.31 ± 0.08 | 1.60 ± 0.04 | –     | 1.41 ± 0.14 | –     | –     | –     | –     |
| TiO2 15 nm         |         |       |       |       |       |       |       |       |       |       |
| Fresh              | 1       | –     | 1.09 ± 0.02 | 1.33 ± 0.11 | –     | 1.19 ± 0.05 | –     | –     | –     | –     |
| 60 days            | 1       | –     | 0.95 ± 0.03 | 1.13 ± 0.06 | –     | 1.32 ± 0.05 | –     | –     | –     | –     |

Cells were treated with graded doses of either different particle sizes of ZnO NPs and TiO2 NPs. Data were pooled from at least three independent experiments and analyzed with one-way ANOVA. Bars: ±S.D. The values in bold are significant (p < 0.05) when compared to 1.31 ± 0.07 (the highest frequency of fresh ZnO 90–200 nm).
fresh NPs (28.09 ± 1.697 and 27.29 ± 0.255 ng). These data indicated that ZnO NPs with smaller size were relatively easily endocytosed by cells leading to the accumulation of Zn element. In contrast, there was no significant difference in the Ti amount between fresh and aged TiO2 NPs-treated cells (Figure 3B).

To identify the respective contribution of soluble and insoluble fractions to the mutagenicity of ZnO NPs, high-performance centrifuge was used for separating metal ions from their solid particulates in the nanoparticle suspensions. The suspensions were centrifuged at 20,000 rpm (28,000 g) for 1 h. As shown in Figure 3(C), both the soluble fraction and the insoluble fraction from fresh and aged ZnO NPs suspension were mutagenic to A549 cells. However, no significant difference of the fold increases of mutation frequencies was observed in cells exposed to fresh ZnO NPs and their insoluble fractions (p > 0.05). With the aging time extended to 60 days, the mutagenicity of aged ZnO NPs was more dependent on the soluble fraction (Zn2+) (p < 0.05). Our results suggested that dissolution showed different contribution to the mutagenesis of fresh and aged ZnO NPs.

Role of Zn2+ on the mutagenicity of fresh and aged ZnO NPs

To better understand the dissolved Zn ions contributing to the mutagenicity of ZnO NPs with aging, the intracellular Zn2+ was determined by Zn-specific fluorescent dye, Newport Green DCF (Cadosch et al., 2009; Xia et al., 2011). Under the confocal microscopy, a visible increase in green fluorescence intensity was mainly located in the cytoplasm of cells exposed to both fresh and aged ZnO NPs. As shown in Figure 4(A), aged ZnO NPs exhibited a higher fluorescence level compared to the fresh ones, indicative of higher intracellular Zn2+ levels. Among the aged ZnO NPs, a relative lower fluorescence was observed in aged NPs with larger size. To investigate the role of Zn2+ in the mutagenicity of ZnO NPs, the metal chelator TPEN (Haase et al., 2009) was used in the present study. Although TPEN was capable of interfering in the mutagenic effects of both fresh and aged ZnO NPs, a significant inhibition effect on the mutation induction was showed in cells exposed to aged ZnO NPs (Figure 4B). With the addition of TPEN, the mutation frequency of aged ZnO NPs 90–200 nm and 20 nm decreased from 1.33 ± 0.028 to 1.165 ± 0.0202 (p = 0.0422) and from 1.6 ± 0.04 to 1.285 ± 0.021 (p = 0.0306), respectively. These results further demonstrated that Zn ions played an essential role in the mutagenicity of ZnO NPs, especially during the aging process. Our data confirmed the contribution of Zn2+ ions, while the emphasis we demonstrated is on the combined contribution of Zn ions and Zn related particulates, and their respective different contributions to the mutagenicity of fresh and aged ZnO NPs.

Figure 3. Contribution of the soluble and insoluble fractions to the mutagenicity of fresh and aged ZnO NPs. Total Zn (A) or Ti (B) element contents in cell samples. ICP-MS analysis to determine Zn or Ti concentration in A549 cells that received 10 μg/mL fresh or 60-day aged ZnO NPs or TiO2 NPs for 72 h. Five million cells were examined in each group in duplicates (p values indicated). Bars: ±S.D. (C) Contributions of the soluble (ions) and insoluble (particulates) fraction on mutagenesis of fresh and 60-day aged ZnO NPs or TiO2 NPs. 10 μg/mL nanoparticles were centrifuged at 20,000 rpm (28,000 g) for 1 h and then the supernatant and precipitant were used to treat A549 cells, respectively. The nanoparticles suspension without centrifugation was used for control. Bars: ±S.D. *Indicated p < 0.05.
Particle uptake and effect of endocytosis inhibitor on the mutagenicity of fresh and aged ZnO NPs

To better determine the contribution of insoluble fraction of NPs solution to the mutagenicity of ZnO NPs with aging, the intracellular location of fresh and aged ZnO NPs was firstly visualized under TEM. The typical TEM images of cells exposed to ZnO NPs are shown in Figure 5(A). A few fresh ZnO NPs were observed and aggregated in the cells, while the aged ZnO NPs were hardly to be found both inside and outside of the treated cells. These observations were consistent with the data on cellular granularity (Figure S2). Overall, there were few ZnO NPs particles (fresh or aged) observed as compared with TiO2 NPs, which were distributed throughout the treated cells (Figure 5A).

Furthermore, Nystatin, an endocytosis inhibitor which disrupts internalization via caveolae (Xu et al., 2009), was used to determine the particle uptake on the mutagenicity of fresh and aged ZnO or TiO2 NPs. As shown in Figure 5(B), the dose of Nystatin used in these experiments was non-mutagenic. The mutation fraction in AL cells induced by fresh ZnO 90–200 nm (or ZnO 20 nm) at a concentration of 10 μg/mL was significantly suppressed in the presence of 10 U/mL Nystatin. With the addition of Nystatin, the mutation frequency of fresh ZnO 90–200 nm and 20 nm decreased from 1.255 ± 0.007 to 0.95 ± 0.141 (p = 0.0098) and from 1.48 ± 0.03 to 1.04 ± 0.028 (p = 0.0041), respectively. The inhibition effects of Nystatin on the mutation yield induced by 60-day aged ZnO NPs treatment were relatively mild. With addition Nystatin, the mutation frequency caused by 60-day aged ZnO 90–200 nm or 20 nm NPs decreased from 1.33 ± 0.028 to 1.195 ± 0.05 (p = 0.0788) or from 1.60 ± 0.04 to 1.40 ± 0.0566 (p = 0.0465), respectively. Our endocytosis inhibition assay indicated that the toxicity of fresh ZnO NPs was mainly contributed from the particulates (ZnO NPs). For aged ZnO NPs, the toxicity were mainly contributed from Zn ions combined with Zn related particulates (Zn5(CO3)2(OH)6). In contrast, the mutation effects of both fresh and aged TiO2 NPs were inhibited almost completely by Nystatin (Figure 5B), which suggested the predominant contribution of the TiO2 NPs particulate-induced mutagenicity. TiO2 NPs did not show obvious dissolution or transformation except for aggregation during aging (Figure 1, Table 1).

Discussion

Engineered NPs in natural systems are subject to a dynamic physical and chemical environment that will drive the particles away from their pristine or “as manufactured” state, toward largely unknown end points and products (Lowry et al., 2012). Aging, as physical or chemical transformations over time, is essential for understanding the fate of NPs in the environment (Lowry et al., 2012; Scheckel et al., 2010). However, related studies focusing on the aged ZnO NPs are limited, especially on the mutagenicity of aged ZnO NPs. In the present study, we found that ZnO NPs underwent dramatical physicochemical transformation with aging, regardless of original morphology or size. The dissolution and transformation of ZnO NPs process might involve the following reactions (Bian et al., 2011; Gelabert et al., 2014; Preis & Gamsjäger, 2001):

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\text{ZnO(s)} + 2\text{H}^+_{(aq)} \rightarrow \text{Zn}^{2+}_{(aq)} + \text{H}_2\text{O} \quad (1)
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\[
\text{Zn}^{2+}_{(aq)} + 0.4\text{CO}_2(g) + 1.6\text{H}_2\text{O(l)} \rightarrow 0.2\text{Zn}_5(\text{CO}_3)_2(\text{OH})_6(s) + 2\text{H}^+_{(aq)} \quad (2)
\]
Figure 5. Particles interact with cell and its contribution to mutagenesis induced by ZnO and TiO$_2$ NPs nanoparticles. (A) Particle uptake detected by TEM following exposure of A$_5$ cells. Cells were incubated with particles (30 µg/mL) for 2 h. Scale bars 5 µm. (B) Mutation fractions at CD59 gene in A$_5$ cells exposed to either fresh or 60-day aged ZnO or TiO$_2$ NPs at a dose of 10 µg/mL either in the presence or absence of Nystatin (10 U/mL), an endocytosis inhibitor. Data were pooled from three independent experiments ($p$ values indicated). Bars: ±S.D.
As seen from the upper equations, the dissolution of ZnO NPs tends to occur under the acidic environment, which was consistent with our finding that with the aging time extended, the pH value increased from 5.5 to 7.2 (Table 1). SAED (Figure S1) and XRD (Figure 1B) analyses verified the formation of Zn$_5$(CO$_3$)$_2$(OH)$_6$. Our results indicated that aging did induce the transformation from ZnO NPs into Zn$^{2+}$ and Zn$_5$(CO$_3$)$_2$(OH)$_6$ in ultrapure water with the existence of CO$_2$. Zinc solid-phase changes from NPs have been reported (Madunkotuwawa et al., 2012). The spontaneous formation of ZnCO$_3$ nanowire species from ZnO nanowires after exposure to ambient air levels of water and CO$_2$ has been observed (Pan et al., 2010b). Modeling of dissolved Zn in moderately hard water by Visual MINTEQ suggests that dissolution of ZnO NPs results in oversaturation of Zn$^{2+}$ with respect to a solid phase of Zn$_5$(CO$_3$)$_2$(OH)$_6$ with possible additional phases of ZnCO$_3$ and ZnO (Reed et al., 2012). Our study confirmed the changes from ZnO NPs into Zn$_5$(CO$_3$)$_2$(OH)$_6$ by multiple characterization methods. This is supported by a recent study conducted by Sivry et al. (2014) who have shown the solid phases obtained after three months of interaction of noncoated ZnO NPs into Seine river water sample were hydrozincite (Zn$_5$(OH)$_6$(CO$_3$)$_2$) and smithsonite (ZnCO$_3$).

The environmental transformations of physicochemical properties in NPs are expected to dramatically alter their toxicity (Rathnayake et al., 2014). It has been reported that sulfidation could decrease the toxicity of ZnO NPs to organisms because the toxicity of ZnO NPs is strongly affected by the release of Zn$^{2+}$ ion (Ma et al., 2013). Since the traditional cytotoxicity assays such as MTT and β-galactosidase assay are largely dependent on the light absorption, the colony formation assay was used in the present study to avoid the potential interference of NPs on the spectrophotometer reading. We found that aged ZnO NPs induced much less cytotoxicity but relatively high mutagenicity than fresh ZnO NPs. The LC$_{100}$ of both fresh ZnO 90–200 nm and 20 nm NPs in our present study was 15 μg/mL, which was consistent with previous reporting that the LC$_{100}$ of ZnO NPs with 19–36 nm to NIH-3T3 or MSTD cell is about 15 μg/mL (Brunner et al., 2006).

Compared to cytotoxicity, which reflects the lethality effects of toxic materials, mutagenicity of NPs could bring much adverse effect to the cell, even the whole organism. The mutagenicity has been considered to be one of the earliest cellular responses caused by physical and chemical carcinogens and may play an important role in the initiation and progression of carcinogenesis (Dixon & Kopras, 2004). Previous studies from this laboratory have shown that the mutation system in A$_L$ cells provides a unique opportunity to assess the mutagenic potential of particulates (Bao et al., 2009). Here, we showed that fresh ZnO 90–200 nm NPs were mutagenic to A$_L$ cells at concentrations $\geq$10 μg/mL ($p<0.05$), which was consistent with the previous studies in bacteria E. coli WP2 trp uvrA and S. typhimurium TA98 and TA1537 stains by Ames test (Kumar et al., 2011; Pan et al., 2010a). Interestingly, we found that after aged for 20, 40 and 60 days, the fold increases of mutation frequencies induced by aged ZnO 90–200 nm NPs at concentrations $\geq$5μg/mL were all higher than that of fresh ZnO NPs (Table 2). Especially, 20 day- and 40 day-aged ZnO 90–200 nm NPs induced 1.90±0.21 and 2.05±0.20 fold increases at 15 and 20 μg/mL, respectively, which were both significantly higher than 1.31±0.07, the highest fold increase of mutation frequency induced by fresh ZnO 90–200 nm NPs. These results suggested that aged ZnO NPs were mutagenic to mammalian cells, even though the lethal effects were significantly decreased with aging time. In contrast, TiO$_2$ 15 nm did not show obvious changes in its mutagenicity with aging.

The roles of dissolution and undissolved parts in the toxicity of ZnO NPs have been studied both in vitro and in vivo (Franklin et al., 2007; Li et al., 2013; Vandenbriel & De Jong, 2012). However, no consistent conclusion has been reached concerning the main contributor to the toxicity of ZnO NPs. It has been reported that the dissolved Zn$^{2+}$ made the main contribution to the cell death in mouse macrophage Ana-1 cells (Song et al., 2010). By contrast, the growth of microorganisms was inhibited by ZnO NPs suspensions, instead of the suspension supernatant, indicating that the toxicity effects were mainly due to ZnO NPs and not due to the dissolved Zn$^{2+}$ (Raghupathi et al., 2011). These conflicting observations may be due to the origin of ZnO NPs transformed in aqueous environment, stability of the stock suspensions, exposure manner and/or the nature of experimental models. A fundamental understanding of the scope and extent of the contribution from both dissolved and undissolved particulates to the toxicity can provide essential information in the potential risk assessment to ecosystem and human beings. In the present study, with characterization of the soluble and insoluble fractions of aged ZnO NPs suspension (Figure 3), together with the control measurements using TPEN (Figure 4) and Nystatin (Figure 5), we revealed that the release of zinc ions and nanoparticle uptake made significantly different contributions to the mutagenicity of fresh and aged ZnO NPs. To further clarify the mechanism of aged ZnO NPs-induced toxicity, we detected the cytotoxicity and mutagenicity of chemicals ZnCl$_2$ and Zn$_5$(CO$_3$)$_2$(OH)$_6$, the two most possible transformed products. As shown in Figure S3, when the concentration $\geq$10 μg/mL, both ZnCl$_2$ and Zn$_5$(CO$_3$)$_2$(OH)$_6$ were cytotoxic and mutagenic to A$_L$ cells, but the cytotoxicity of both ZnCl$_2$ and Zn$_5$(CO$_3$)$_2$(OH)$_6$ were much less than that of fresh ZnO NPs. In contrast, the mutation frequencies induced by ZnCl$_2$ and Zn$_5$(CO$_3$)$_2$(OH)$_6$ remained comparable high level with that of fresh ZnO NPs (Table 2, Figure S3B and D). With these results, we deduced that the higher cytotoxicity of fresh ZnO NPs could be mainly due to the intrinsic toxicity of ZnO NPs, while the higher mutation potential of aged ZnO NPs might be attributed to the main contribution of Zn$^{2+}$ and the lesser contribution from the major transformed products, i.e. Zn$_5$(CO$_3$)$_2$(OH)$_6$.

Conclusions

This paper demonstrated that ZnO NPs underwent sophisticated physicochemical transformations with aging regardless of original morphology or size, such as the microstructural changes, the formation of hydrozincite (Zn$_5$(CO$_3$)$_2$(OH)$_6$) and the release of free zinc ions. The physicochemical transformations of ZnO NPs were well correlated with the difference in toxic effects between fresh and aged ZnO NPs. Although the cytotoxicity of ZnO NPs declined dramatically with aging, the aged ZnO NPs resulted in relatively high degree mutation in A$_L$ cells. After separating the soluble and insoluble fractions of fresh and aged ZnO suspension by centrifugation, together with the control measurements using metal chelator and endocytosis inhibitor, the mutation induction of aged ZnO NPs was found to be closely related to the soluble fraction and partially mediated by the transformed particulates. Our findings suggest that the physicochemical transformation of ZnO NPs with aging plays important and comprehensive roles in the mutagenicity in mammalian cells, which provides new insights into the health risk assessment on ZnO NPs. Although whether there is difference in the molecular mechanism between fresh and aged ZnO NPs is still unclear, we believe that the results reported in this paper provide us with a reference for the overall toxicity assessment. Still much work is required to be carried out to analyze the real state, behavior and toxicity of NPs in environment and to elucidate the mechanism of how NPs induce the mutagenicity.
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Declaration of interest

The authors declare no competing financial interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online
Supplementary Figures S1–S3.