The Journal of Toxicological Sciences (J. Toxicol. Sci.)
Vol.34, No.1, 119-122, 2009

Letter

Mutagenicity of water-soluble ZnO nanoparticles in Ames test

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(Received October 23, 2008; Accepted November 12, 2008)

ABSTRACT — A mutagenicity test was conducted on water-soluble ZnO nanoparticles capped with tetramethylammonium hydroxide in a bacterial reverse mutation assay using *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537, and *Escherichia coli* strain WP2*uvrA*$^-$, with and without metabolic activation by S9 mix in the preincubation method. Mutagenicity was negative in all strains.

Key words: ZnO nanoparticle, Tetramethylammonium hydroxide, Mutagenicity, Toxicity, Ames test, Bacterial reverse mutation test

INTRODUCTION

Zinc oxide (ZnO) nanoparticles (NPs) are an excellent oxide semiconductor material for n-type thin-film transistors and phosphors because of their superior electric and optical properties (Sun and Sirringhaus, 2005). Meanwhile, ZnO NPs are also expected to be used as a material for next-generation transparent electrodes (Zhang *et al.*, 2005). Hence, the importance of dispersion of ZnO (ZnO ink) is rising recently. To utilize ZnO NPs industrially, their safety must be strictly confirmed. There is concern that nano-sized materials exhibit unknown biological or environmental effects, even if their bulk counterparts are known to be safe. It is thus an urgent issue to test the safety (or hazard) of nanomaterials on a global basis (Oberdörster *et al.*, 2004; Hardman, 2006).

There are various tests to study the safety of chemicals. The bacterial reverse mutation test (Ames test) is a simple biological assay to assess the mutagenic potential of chemicals (Ames *et al.*, 1972, 1973; McCann *et al.*, 1975), which has been widely used in the screening of chemicals. In this study, the mutagenicity of ZnO NPs capped with tetramethylammonium hydroxide (TMAOH) was investigated by a modified Ames test (Wilcox *et al.*, 1990), because TMAOH-capped ZnO NPs are water-soluble. Water solubility is quite important for industrial applications of ZnO NPs. Recently, Dufour *et al.* (2006) investigated the clastogenicity of uncoated ZnO submicron particles (mean particle size, 100 nm) in Chinese hamster ovary with or without UV irradiation to clarify whether the photoinduced enhancement effect of clastogenic potency is a genuine photo-genotoxic effect (Dufour *et al.*, 2006). Their results suggest that minor increases in clastogenic potency under conditions of photo-genotoxicity testing do not necessarily represent a photo-genotoxic effect, but may occur due to an increased sensitivity of the test system subsequent to UV irradiation (Dufour *et al.*, 2006). Someya *et al.* (2008) have reported that chromosome aberrations in human dental pulp cells were induced by ZnO powder. However, they concluded that the results of genetic-toxicity tests for zinc were equivocal and might be dependent on the zinc compounds tested and the type of cells used (Someya *et al.*, 2008). It is highly possible that the size of ZnO particles also relates to genotoxicity.

MATERIALS AND METHODS

Preparation of TMAOH-capped ZnO NPs

ZnO NPs were synthesized using a previously reported method (Sun and Sirringhaus, 2005) with some modifications. Briefly, a 4.46 mmol of zinc acetate [$Zn(Ac)_2$] (Sigma-Aldrich, St. Louis, MO, USA), 25 µl of pure water, and 42 ml of methanol (Kanto Chemical, Tokyo, Japan) were placed in a three-necked flask and the mixture agitated for 5 min under an Ar atmosphere. Subsequently, the temperature was raised to 60°C. Then, 23 ml of methanol solution of KOH (314 mM) was added into the mixture dropwise. After 120 min of reaction at 60°C with reflux, ZnO NPs were separated from the matrix by

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centrifugation. ZnO NPs were characterized by transmission electron microscopy (TEM) and X-ray diffractometry (XRD) (Fig. 1). The mean diameter and standard deviation of the size distribution were found to be 5.4 nm and 15%, respectively. Crystal structure was wurtzite. As-synthesized ZnO NPs were capped with acetic acid and well-dispersed in nonpolar solvents.

Ligand exchange from acetic acid to TMAOH was carried out following a method described in the literature (Salgueiriño-Maceira *et al.*, 2004) (Fig. 2). A TMAOH aqueous solution (25wt%, Wako Pure Chemical, Osaka, Japan) was added to ZnO NPs at the rate of 1 ml per 10 mg of NPs; then the mixture ultrasonically agitated for several minutes. After sonication, the ZnO NP dispersion was centrifuged. Subsequently, TMAOH solution was added to the precipitate (ZnO NPs) and the NP dispersion was centrifuged once again. The precipitate was then completely discarded. As a consequence, we obtained an aqueous dispersion of TMAOH-capped ZnO NPs at a solid concentration of 5wt% (stock dispersion). We prepared a 10 ml stock dispersion. Note that no aggregation was observed by dynamic light scattering and TEM in the stock dispersion.

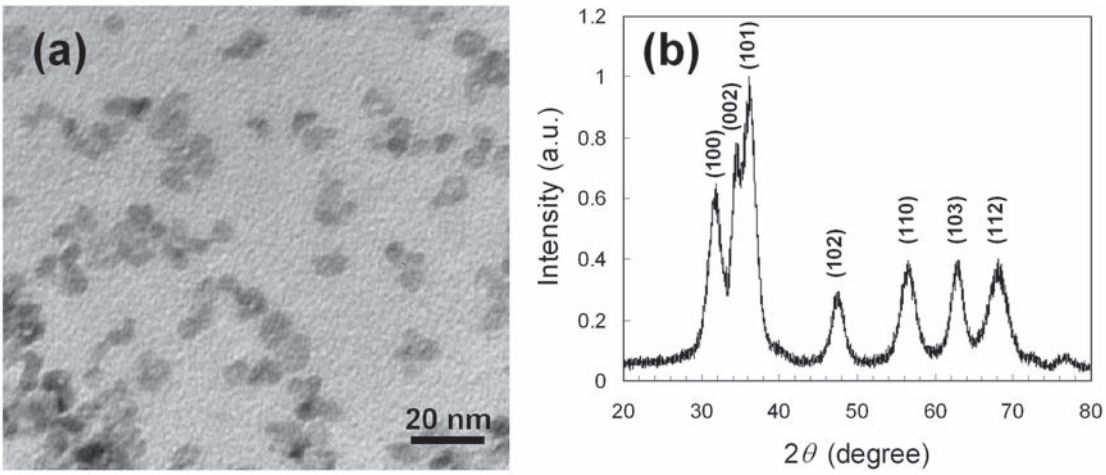

Fig. 1. (a) Transmission electron microscope image of as-synthesized ZnO NPs with a mean diameter 5.4 nm. (b) XRD pattern of ZnO NPs indicating that their crystal structure is wurtzite.

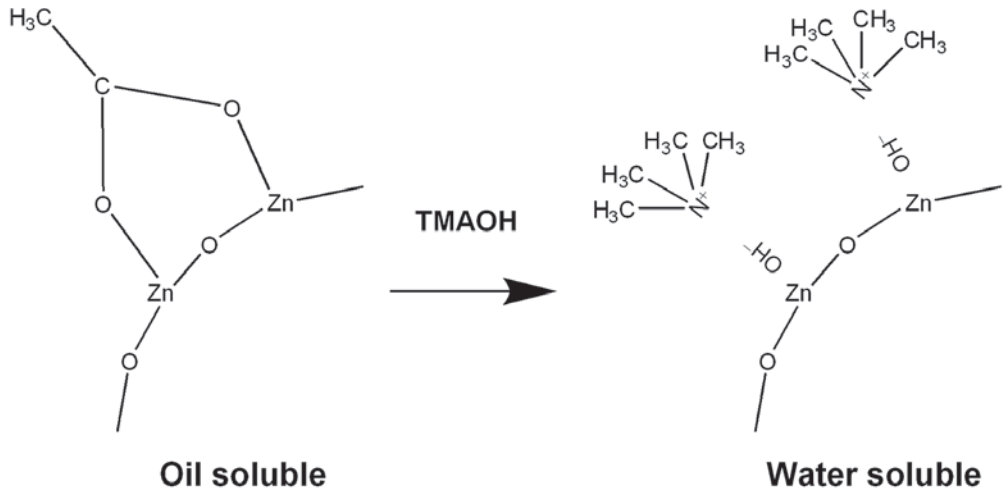

Fig. 2. Schematic illustration of ligand exchange

Bacterial mutagenicity test

The tester strains used in this study were *S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2*uvrA*⁻, provided by the Japan Bioassay Research Center. The culture stocks were stored below −80°C. The tester strain was freshly prepared by pre-culturing for 8 hr at 37°C in nutrient broth (Oxoid No.2). Dimethyl sulfoxide (0.7 ml; Wako Pure Chemical) was added to 8.0 ml of the dispersion of the tester strain.

Male rat liver S9 (Sprague-Dawley) pretreated with phenobarbital/5,6-benzoflavone was purchased from Kikkoman Corp. (Chiba, Japan) Cofactor mix was prepared by adding 9 ml of sterile distilled water to Cofactor-I (Oriental Yeast, Tokyo, Japan) and filtrating the cofactor solution. S9 mix (1 ml) contained 0.1 ml of S9 fraction and 0.9 ml of Cofactor mix that contains Cofactor, $MgCl_2$, KCl, D-glucose-6-phosphate, β-NADPH, β-NADH, and sodium phosphate. Thus, 1 ml of S9 mix contained 0.1 ml of S9, 8 μmol of $MgCl_2$, 33 μmol of KCl, 5 μmol of D-glucose-6-phosphate, 4 μmol of β-NADPH, 4 μmol of β-NADH and 100 μmol of sodium phosphate (pH 7.4).

The mutagenicity test was conducted using a preincubation assay (Yahagi *et al.*, 1977). The tester strains were incubated with nutrient broth and reaction mixture containing phosphate buffer/S9 mix, the tester strain and TMAOH-capped ZnO NPs for 8 hr at 37°C with shaking at 80 strokes per minute. After incubation, top agar was added to the mixture, which was then poured onto a plate of minimal glucose agar medium. The plate was incubated for 48 hr at 37°C and revertant colonies that appeared were counted. Two plates were used for each dose and an average value was calculated. The positive control used during −S9 mix was 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2) for TA98 and TA100 strains, *N*-Ethyl-*N*'-nitro-*N*-nitrosoguanidine (ENNG) for the WP2*uvrA*⁻ strain, sodium azide (NaN_3) for the TA1535 strain, and 9-aminoacridine hydrochloride (9-AA) for the TA1537 strain. The positive control used during +S9 mix was 2-aminoanthracene (2-AA) for all tester strains.

RESULTS AND DISCUSSION

Mixtures of TMAOH-capped ZnO NPs and excess TMAOH molecules were tested for bacterial mutagenicity using the *S. typhimurium* strains TA98, TA100, TA1535, TA1537, and the *E. coli* strain WP2*uvrA*⁻. The amounts of TMAOH-capped ZnO NPs with excess TMAOH molecules used were 39.1 μg, 78.1 μg, 156 μg, 313 μg, 625 μg, 1,250 μg, 2,500 μg or 5,000 μg per plate. Because the molecular weight of ZnO NP is quite large, the dose of ZnO NPs would be high compared with that of positive controls. All experimental results are summarized in Table 1. Growth inhibition was observed in all test-

Table 1. The numbers of total colonies including spontaneous revertant colonies that appeared on a plate

Dose (mg/plate)	Base-pair substitution type						Frameshift type			
	TA100		TA1535		WP2uvrA-		TA98		TA1537	
	−S9mix	+S9mix	−S9mix	+S9mix	−S9mix	+S9mix	−S9mix	+S9mix	−S9mix	+S9mix
0	126 ± 2	136 ± 0	12 ± 3	9 ± 1	31 ± 4	37 ± 0	19 ± 3	24 ± 4	10 ± 1	10 ± 1
39.1	120 ± 14	123 ± 5	7 ± 1	11 ± 1	30 ± 4	36 ± 4	16 ± 1	26 ± 4	7 ± 1	10 ± 3
78.1	126 ± 4	119 ± 8	7 ± 1	8 ± 0	29 ± 2	41 ± 0	18 ± 3	28 ± 4	7 ± 1	8 ± 0
156	137 ± 6	109 ± 5	11 ± 1	8 ± 1	33 ± 4	35 ± 1	24 ± 4	26 ± 4	8 ± 1	12 ± 2
313	130 ± 20	122 ± 4	8 ± 1	6 ± 1	39 ± 1	35 ± 1	19 ± 0	22 ± 1	8 ± 1	13 ± 1
625	143 ± 6	105 ± 2*	7 ± 3	7 ± 1*	34 ± 8	36 ± 6	23 ± 4	28 ± 3*	8 ± 1	12 ± 2*
1,250	81 ± 1*	81 ± 4*	6 ± 2*	0 ± 0*	17 ± 5*	17 ± 4*	11 ± 3*	17 ± 5*	3 ± 1*	10 ± 3*
2,500	0 ± 0*	0 ± 0*	0 ± 0*	0 ± 0*	0 ± 0*	0 ± 0*	0 ± 0*	0 ± 0*	0 ± 0*	0 ± 0*
5,000	0 ± 0*	0 ± 0*	0 ± 0*	0 ± 0*	0 ± 0*	0 ± 0*	0 ± 0*	0 ± 0*	0 ± 0*	0 ± 0*
Positive	950 ± 35 (0.01)	864 ± 30 (1.0)	434 ± 12 (0.5)	308 ± 16 (2.0)	632 ± 29 (2.0)	757 ± 82 (10)	557 ± 12 (0.1)	432 ± 4 (0.5)	311 ± 98 (80)	134 ± 12 (2.0)

The negative control was sterile distilled water. The positive control used during −S9 mix was AF-2 for TA98 and TA100 strains, ENNG for the WP2uvrA- strain, NaN3 for the TA1535 strain, and 9-AA for the TA1537 strain; during +S9 mix 2-AA was used for all tester strains. Asterisks (*) represent that growth inhibition was observed. Values in parentheses correspond to the doses of positive control chemicals (mg/plate).

er strains with or without S9 mix due to the addition of TMAOH-capped ZnO NPs in cases of high-dose. Large increases in the number of revertant colonies were seen for the positive controls in all cases (Table 1), indicating that the test system responded appropriately. However, mutagenicity was negative in all strains with or without S9 mix, as shown in Table 1.

The mutagenicity of TMAOH was previously tested using the *S. typhimurium* strains TA98, TA100, TA1535 and TA1537, and the *E. coli* strain WP2*uvrA*, with and without S9 mix. As a result, the mutagenicity of TMAOH was found to be negative in all tester strains (Biosafety Research Center, Foods, Drugs and Pesticides, 1999). Hence, the mutagenicity of bare ZnO NPs is considered to be negative.

In conclusion, the mutagenicity test was conducted on ZnO NPs capped with TMAOH using the bacterial reverse mutation assay. Mutagenicity was found to be negative in all strains with or without S9 mix. Further detailed toxicological investigations, such as a micronucleus assay, are necessary to determine the genotoxicity of ZnO NPs.

ACKNOWLEDGMENTS

We thank Mr. M. Oda, Ms. E. Ishii, Ms. A. Seo, and Ms. Y. Sakurai from Japan Oil Stuff Inspectors' Corporation for the bacterial reverse mutation assay. This work is supported by a Grant for Industrial Technology Research Program in 2006 from New Energy and Industrial Technology Development Organization (NEDO) of Japan.

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