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Description	

– Letter –

MUTAGENICITY OF WATER-SOLUBLE FePt
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ABSTRACT — A mutagenicity test was conducted on water-soluble FePt nanoparticles capped with tetramethylammonium hydroxide in a bacterial reverse mutation assay using *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537, and *Escherichia coli* strain WP2uvrA/pKM101, with and without metabolic activation by S9 mix in the preincubation method. Mutagenicity was weakly positive in the TA100 strain without S9 mix (maximum specific activity was 61.6 revertants/mg), but negative in other cases.

KEY WORDS: FePt nanoparticle, Tetramethylammonium hydroxide, Mutagenicity, Toxicity, Ames test, Bacterial reverse mutation test

INTRODUCTION

Iron-platinum (FePt) nanoparticles (NP) are an excellent magnetic material for ultra-high density magnetic storage media because of their superior magnetic properties (Sun *et al.*, 2000; Sun, 2006). Meanwhile, FePt NPs are also expected to be a high-performance nanomagnet for magnetic medicine, such as magnetic hyperthermia (Maenosono and Saita, 2006), magnetic resonance imaging (Zhao *et al.*, 2001), immunomagnetic cell separation (Gu *et al.*, 2003), and magnetofection (Dobson, 2006), because it presents a high Curie temperature, high saturation magnetization and high chemical stability. However, to utilize FePt NPs in the field of medicine, their safety must be strictly confirmed. Obviously, the environment safety of FePt NPs should also be investigated when they are utilized in device applications, such as hard-disk media. There is concern that nano-sized materials exhibit unknown biological or environmental effects, even if their bulk counterparts are known to be safe. Hence, it is 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, including acute toxicity tests, subacute toxicity tests, chronic toxicity tests, carcinogenesis tests, mutagenicity tests, neurotoxicity tests, immunotoxicity tests, inhalation toxicity tests, dermatotoxicity tests and ecotoxicologic tests. The bacterial reverse mutation test, which was developed by Ames *et al.*, is a simple biological assay to assess the mutagenic potential of chemicals (Ames test) (Ames *et al.*, 1972, 1973; McCann *et al.*, 1975). The Ames test has been widely used in the screening of chemicals as a preliminary screen for cancer bioassays and to eliminate substances that show strong mutagenicity. In this study, the mutagenicity of FePt NPs capped with tetramethylammonium hydroxide (TMAOH) was investigated by a modified Ames test (Wilcox *et al.*, 1990), using five tester strains: *Salmonella typhimurium* (*S. typhimurium*) TA98, TA100, TA1535 and TA1537, and *Escherichia coli* (*E. coli*) WP2uvrA/pKM101, because TMAOH-capped FePt NPs are water-soluble and because the mutagenicity of TMAOH itself was found to be negative (Biosafety Research Center, Foods, Drugs and Pesticides, 1999).

MATERIALS AND METHODS

Preparation of TMAOH-capped FePt NPs

FePt NPs were synthesized using a previously reported method (Saita and Maenosono, 2005) with some modifications. Briefly, a 2.0 mmol of iron(III)ethoxide [$\text{Fe}(\text{OEt})_3$] (Alfa Aesar), 0.5 mmol of platinum(II)acetylacetonate [$\text{Pt}(\text{acac})_2$] (Aldrich), 1.27 mL of oleic acid (Aldrich), and 6.58 mL of oleylamine (Aldrich) were placed in a three-necked flask and the mixture was ultrasonically agitated for 5 min. Subsequently, the temperature was raised to 250°C under an Ar atmosphere. After 30 min of reaction at 250°C, FePt NPs were separated from the matrix by centrifugation. FePt NPs were characterized by transmission electron microscopy, X-ray diffractometry, energy dispersive X-ray analysis, FT-IR, CHNS elemental analyzer and SQUID. The mean diameter, standard deviation of the size distribution, crystal structure, composition, surface ligand and magnetocrystalline anisotropy energy were found to be 9 nm (Fig. 1a), 11%, face-centered cubic, $\text{Fe}_{50}\text{Pt}_{50}$, oleic acid, and 130 kJ/m^3 , respectively.

Ligand exchange from oleic acid to TMAOH was carried out following a method described in the literature (Salgueiriño-Maceira *et al.*, 2004). Six mL of 15wt% TMAOH (Wako Pure Chemical) was added to

150 mg of FePt NPs; then, the mixture was ultrasonically agitated for 20 min. After sonication, 3 mL of the dispersion was poured into acetone (30 mL). Subsequently, 3 mL of ethanol and 3 mL of pure water were added into the dispersion and the dispersion was centrifuged. The supernatant was then completely discarded. The weight of the precipitate was 12 mg. Finally, the precipitate was redispersed in 228 mg of a diluted TMAOH aqueous solution (5wt%). As a consequence, we obtained an aqueous dispersion of TMAOH-capped FePt NPs at a solid concentration of 5wt% (stock dispersion). We prepared an 11.4 g stock dispersion. FT-IR spectroscopy confirmed that oleic acid was successfully replaced with TMAOH, as shown in Fig. 1b.

Bacterial mutagenicity test

The tester strains used in this study were *S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2uvrA/pKM101, 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

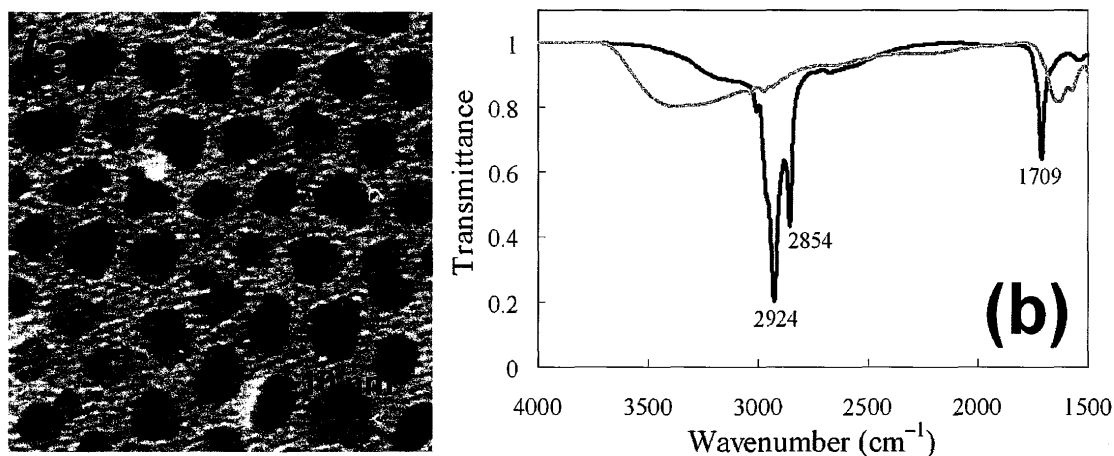


Fig. 1. (a) Transmission electron microscope image of as-synthesized FePt NPs with a mean diameter 9 nm. (b) IR spectra of FePt NPs capped with oleic acid (black line) and with TMAOH (gray line) taken by diffuse reflectance FT-IR spectroscopy. Peaks at 2854 and 2924 cm^{-1} correspond to the symmetric and asymmetric CH_2 stretching modes of the oleyl group, respectively. The characteristic COOH group peak at 1713 cm^{-1} suggests that free oleic acid molecules remain in the sample. These characteristic peaks of oleic acid disappear after the ligand exchange reaction (gray line), indicating that oleic acid was successfully replaced with TMAOH.

Mutagenicity of water-soluble FePt nanoparticles in Ames test.

with phenobarbital/5,6-benzoflavone was purchased from Kikkoman Corp. Cofactor mix was prepared by adding 9 mL of sterile distilled water to Cofactor-I (Oriental Yeast) and filtrating the cofactor solution. S9 mix (1 mL) contained 0.1 mL of S9 fraction and 0.9 mL of Cofactor mix. Thus, 1 mL of S9 mix contained 0.1 mL of S9, 8 mmol of MgCl_2 , 33 mmol of KCl, 5 mmol of D-glucose-6-phosphate, 4 mmol of b-NADPH, 4 mmol of b-NADH and 100 mmol of sodium phosphate (pH 7.4).

Top agar was prepared by dissolving 0.6 g of Bacto-agar (Becton Dickinson and Company) and 0.5 g of NaCl (Wako Pure Chemical) in 100 mL of purified water and sterilizing the solution for 15 min at 121°C using an autoclave. The top agar was stored at 45°C. For the culture of *S. typhimurium*, a mixed aqueous solution of L-histidine (0.5 mmol/L) and D-biotin (0.5 mmol/L) was added to the agar medium until shortly before use (tenth part of the agar solution). For the culture of *E. coli*, an aqueous solution of L-tryptophan (0.5 mmol/L) was added to the agar medium (tenth part of the agar solution).

The mutagenicity test was conducted using a pre-incubation 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 the TMAOH-capped FePt 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, TA100 and WP2uvrA/pKM101 strains, 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 FePt NPs and excess TMAOH molecules were tested for bacterial mutagenicity using the *S. typhimurium* strains TA98, TA100, TA1535, TA1537, and the *E. coli* strain WP2uvrA/pKM101. The concentrations of TMAOH-capped FePt NPs with excess TMAOH molecules used were 78.1 µg, 156 µg, 313 µg, 625 µg, 1250 µg, 2500 µg or 5000 µg per plate. All experimental results are summarized in Table 1. No growth inhibition was observed in any tester strain with or without S9 mix due to the addition of TMAOH-capped FePt NPs, regardless of dose. However, mutagenicity was weakly positive in the TA100 strain without S9 mix, as shown in Fig. 2a. The maximum specific activity was calculated to be 61.6 revertants/mg (dose 2500 µg/plate). On the other hand, mutagenicity was negative in the TA100 strain with S9 mix.

The mutagenicity of TMAOH was previously

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

Dose (µg/plate)	Base-pair substitution type						Frameshift type			
	TA100		TA1535		WP2uvrA/ pKM101		TA98		TA1537	
	-S9 mix	+S9 mix	-S9 mix	+S9 mix	-S9 mix	+S9 mix	-S9 mix	+S9 mix	-S9 mix	+S9 mix
0	121 ± 6	130 ± 2	9 ± 1	10 ± 1	81 ± 2	101 ± 5	24 ± 2	27 ± 1	9 ± 1	10 ± 1
78.1	131 ± 1	137 ± 5	12 ± 2	12 ± 2	75 ± 2	99 ± 7	28 ± 4	27 ± 4	10 ± 1	13 ± 2
156	140 ± 6	143 ± 1	9 ± 1	9 ± 1	78 ± 3	103 ± 17	27 ± 4	28 ± 2	9 ± 1	13 ± 1
313	150 ± 13	140 ± 6	11 ± 1	10 ± 1	85 ± 3	116 ± 4	23 ± 4	32 ± 3	10 ± 2	12 ± 2
625	178 ± 7	147 ± 13	9 ± 1	13 ± 2	76 ± 1	108 ± 1	30 ± 1	27 ± 2	11 ± 1	12 ± 0
1250	213 ± 0	168 ± 7	11 ± 3	10 ± 1	91 ± 8	103 ± 6	30 ± 7	35 ± 7	10 ± 1	11 ± 3
2500	275 ± 10	166 ± 7	13 ± 0	12 ± 4	86 ± 8	112 ± 8	43 ± 3	37 ± 7	8 ± 0	13 ± 3
5000	329 ± 4	189 ± 9	14 ± 1	12 ± 1	89 ± 8	102 ± 4	41 ± 2	38 ± 6	12 ± 3	13 ± 2
Positive	771 ± 2	1207 ± 23	508 ± 76	315 ± 13	1027 ± 59	897 ± 22	737 ± 24	537 ± 42	469 ± 35	244 ± 5
	(0.01)	(1.0)	(0.5)	(2.0)	(0.005)	(2.0)	(0.1)	(0.5)	(80)	(2.0)

The negative control was sterile distilled water. The positive control used during -S9 mix was AF-2 for TA98, TA100 and WP2uvrA/pKM101 strains, NaN_3 for the TA1535 strain, and 9-AA for the TA1537 strain; during +S9 mix 2-AA was used for all tester strains. Values in parentheses correspond to the doses of positive control chemicals (µg/plate).

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

This result indicates that the rat liver microsomal enzyme system somehow inactivates the mutagenic property of TMAOH-capped FePt NPs. As shown in Figs. 2b-e, mutagenicity was negative in TA98,

TA1535, TA1537 and WP2*uvrA*/pKM101 strains, with or without S9 mix. Mutagenicity observed in TA100 without S9 mix might be connected to the previous results of a cytotoxicity test of FePt NPs (Kim *et al.*, 2005). According to Kim *et al.* (2005), uncoated Fe₄₈Pt₅₂ NPs had no significant toxicity on bEnd3 cells in a 24 hr period when the NP concentration was not so high, but showed cell cytotoxicity when the concentration was relatively high.

In conclusion, the mutagenicity test was conducted on FePt NPs capped with TMAOH using the bacterial reverse mutation assay. Mutagenicity was

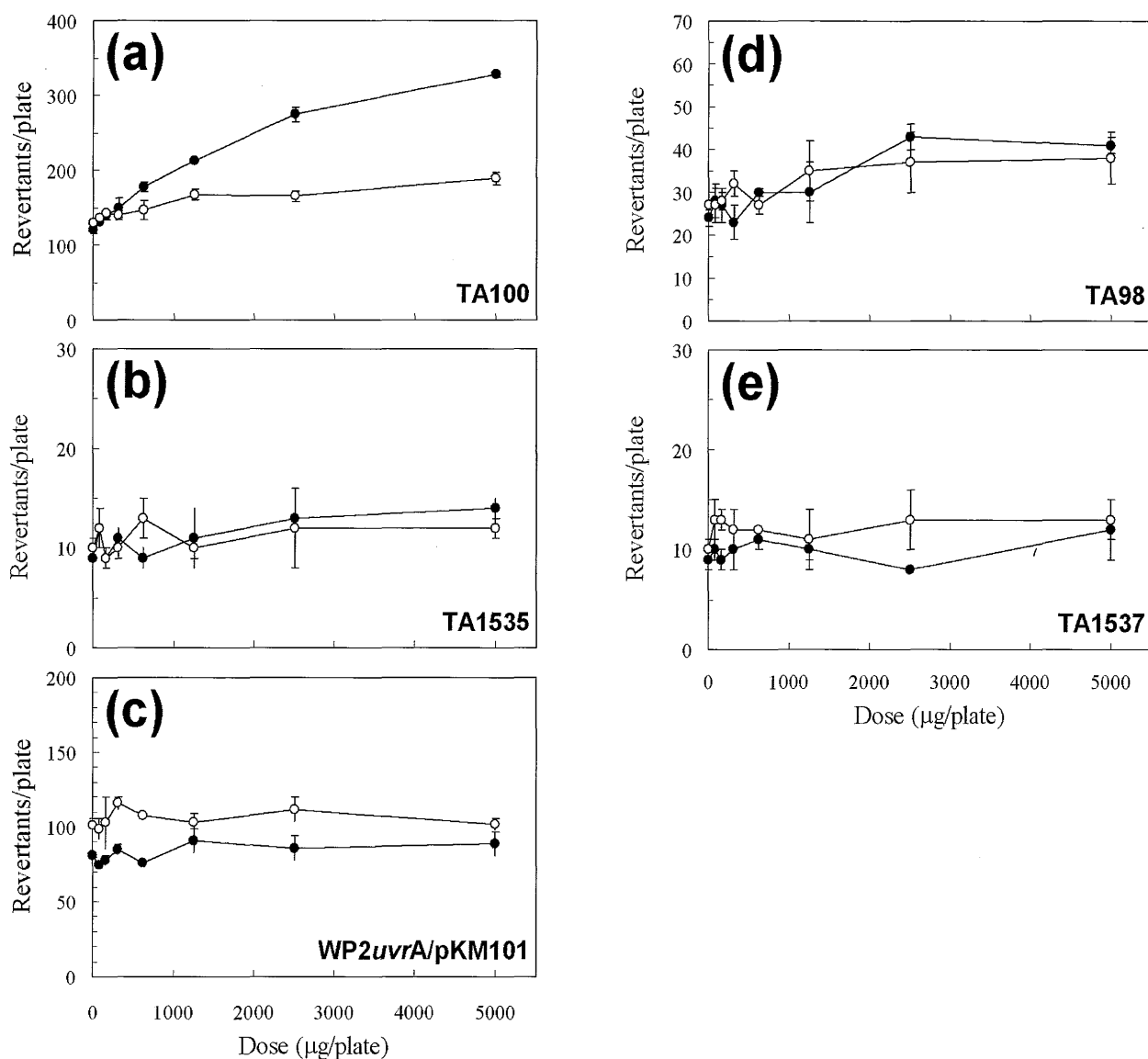


Fig. 2. Dose-response curves of the TMAOH-capped FePt NPs on (a)TA100, (b)TA1535, (c) WP2 *uvrA*/pKM101, (d) TA98 and (e) TA1537 strains. Open and filled circles correspond to experimental data obtained with and without S9 mix, respectively.

Mutagenicity of water-soluble FePt nanoparticles in Ames test.

found to be weakly positive in the TA100 strain without S9 mix, while it was negative in other cases. Further detailed toxicological investigations, such as a micronucleus assay, are necessary to determine the genotoxicity of FePt NPs.

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