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Letter

Evaluation of genotoxicity of amine-terminated water-dispersible FePt nanoparticles in the Ames test and *in vitro* chromosomal aberration test

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ABSTRACT — Genotoxicity of superparamagnetic iron-platinum (FePt) nanoparticles (NPs) capped with 2-aminoethanethiol (AET) was evaluated using the bacterial reverse mutation assay (Ames test) and *in vitro* chromosomal aberration test. Mutagenicity of AET-capped FePt NPs was found to be negative in the Ames test, while clastogenicity of FePt NPs seemed to be false-positive in the *in vitro* chromosomal aberration test using Chinese hamster lung fibroblast cells. However, further detailed *in vitro* genotoxicity tests, such as DNA adduct studies, are necessary to conclude that a positive aberration result is irrelevant.

Key words: FePt nanoparticle, 2-aminoethanethiol, Cysteamine, Genotoxicity, Bacterial reverse mutation assay, Chromosomal aberration test

INTRODUCTION

Iron-platinum (FePt) nanoparticles (NPs) 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). Recently, we investigated the mutagenicity of water-dispersible FePt NPs capped with tetramethylammonium hydroxide (TMAOH) in the Ames test. In consequence, the mutagenicity of TMAOH-capped FePt NPs was found to be weakly positive, though the mutagenicity of TMAOH itself was negative (Maenosono *et al.*, 2007). This result suggests that the mutagenicity is considered to be caused by FePt NPs or FePt NP/TMAOH complexes.

In this study, the genotoxicity of FePt NPs capped with 2-aminoethanethiol (AET; aliases cysteamine, mercaptamine, or becaptan) 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*) WP2*uvrA*$^-$, and by a chromosomal aberration test, using Chinese hamster lung fibroblast (CHL/IU) cells, because AET-capped FePt NPs have positive surface charge that is important for biological applications, and because AET are known to exhibit antimutagenic effects against chemical mutagens (Hartman and Shankel, 1990; Hoffmann *et al.*, 1999).

MATERIALS AND METHODS

Synthesis of FePt NPs

FePt NPs were synthesized using a previously report-

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ed method (Kang *et al.*, 2008) with some modifications. Briefly, 0.38 mmol of triiron dodecacarbonyl [$Fe_3(CO)_{12}$] (CAS registry number 17685-52-8; Sigma-Aldrich) was dissolved in 37.5 ml of hexane under a nitrogen atmosphere. Then, 0.72 ml of oleic acid (CAS registry number 2027-47-6; Sigma-Aldrich, St. Louis, MO, USA) was added to the solution. Meanwhile, 100 mg of platinum(II) acetylacetonate [$Pt(acac)_2$] (CAS registry number 15170-57-7; Sigma-Aldrich), 0.87 ml of oleylamine (CAS registry number 112-90-3; Sigma-Aldrich), and 15 ml of diethylene glycol (CAS registry number 111-46-6; Sigma-Aldrich) were placed in a three-necked flask and the mixture was agitated for 5 min under an Ar atmosphere. Subsequently, the temperature was raised to 80°C. Then, the $Fe_3(CO)_{12}$/hexane solution was rapidly injected into the flask, and the reaction mixture was stirred for 30 min at 80°C to remove volatile matter. Subsequently, the temperature was raised to 240°C. After 2 hr of refluxing, FePt NPs capped with oleic acid were separated from the matrix by centrifugation. FePt NPs were characterized by transmission electron microscopy (TEM) and X-ray diffractometry (XRD). The mean diameter, standard deviation of the size distribution, and crystal structure were found to be 3 nm (Fig. 1), 14.5%, and face-centered cubic, respectively.

Ligand exchange

Ligand exchange from oleic acid to AET was carried out following a method described in the literature (Tanaka and Maenosono, 2008). Thirteen ml of chloroform (Kanto Kagaku) and 15.8 ml of methanol solution of AET hydrochloride (0.5 M; CAS registry number 156-57-0; Wako Pure Chemical, Osaka, Japan) were added to 75 mg of FePt NPs; then, the mixture was ultrasonically agitated for 10 min. After sonication, 15.8 ml of pure water was poured into the dispersion; then the dispersion was agitated for 5 min. Subsequently, the dispersion was centrifuged and the supernatant was then completely discarded. Finally, the precipitate was redispersed in pure water. As a consequence, we obtained an aqueous dispersion of AET-capped FePt NPs at a solid concentration of 5 wt% (stock dispersion). We prepared a 10 ml stock dispersion. Note that no aggregation was observed in the stock dispersion.

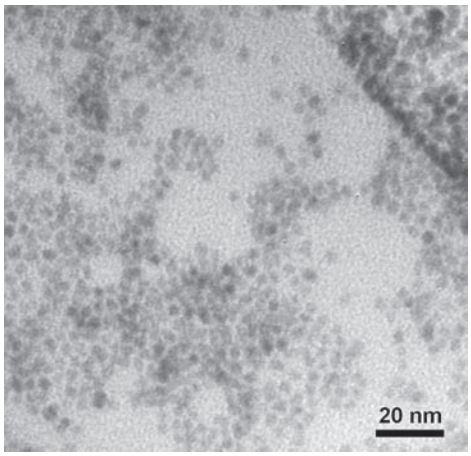

Fig. 1. TEM image of AET-capped FePt NPs with a mean diameter 3 nm.

Ames test

The test was conducted according to standard procedures following OECD guidelines. 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. Male rat liver S9 (Sprague-Dawley) pretreated with phenobarbital/5,6-benzoflavone was purchased from Kikkoman Corp. (Chiba, Japan). 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 (Oxoid No.2, 10 ml) and reaction mixture containing 0.1 M phosphate buffer/S9 mix (1 ml) and the AET-capped FePt NPs for 8 hr at 37°C. After incubation, top agar (100 ml) 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.

In vitro chromosomal aberration test

The test was conducted according to standard procedures following OECD guidelines. CHL/IU cells were purchased from DS Pharma Biomedical Co., Ltd. (Osa-

ka, Japan). The assay consisted of short-term and continuous (24 hr) treatments at three or more dose levels. The maximum dose of AET-capped FePt NPs was selected from the doses at which > 50% cell growth inhibition was observed in a preliminary test. For the short-term treatment, AET-capped FePt NPs were administered for 6 hr followed by a recovery period of 18 hr. For the metabolic activation, the cells were treated with the above-mentioned S9 mix (0.5 ml) together with FePt NPs (0.3 ml). Both with and without the metabolic activation system, the cells were treated with colcemid (0.1 μg/ml) for 2 hr, and chromosome preparations were made using a standard air-dry method (Miyake *et al.*, 2000). The positive control used during −S9 mix was mitomycin C (MMC). The positive control used during +S9 mix was benzopyrene (BP). The frequency of the cells with structural and numeric chromosomal aberrations was scored in 100 well-spread metaphases for each dose. Types of structural chromosomal aberrations were classified into six groups: chromatid and chromosome gaps (gap), fragmentation (frg), chromatid breaks (ctb), chromatid exchanges (cte), chromosome breaks (csb) and chromosome exchanges (cse) including dicentric and ring chromosomes. Polyploid cells were also recorded. The final result of AET-capped FePt NPs was judged as follows: negative (−) if the frequency of aberrant cells was < 5%, inconclusive (±) if ≥ 5% but < 10%, and positive (+) if ≥ 10%.

RESULTS AND DISCUSSION

Ames test

Aqueous dispersions of AET-capped FePt NPs were tested for bacterial mutagenicity using the *S. typhimurium* strains TA98, TA100, TA1535, TA1537, and the *E. coli* strain WP2*uvrA*$^-$. The concentrations of AET-capped FePt NPs 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. All experimental results are summarized in Table 1. No growth inhibition was observed in any tester strains with or without S9 mix due to the addition of AET-capped FePt NPs, regardless of dose. The presence of precipitation of the NPs was observed at dose of 5,000 μg/plate with or without S9 mix. Mutagenicity was negative in all strains with or without S9 mix, as shown in Table 1.

Recently, we investigated the mutagenicity of TMAOH-capped FePt NPs in the bacterial reverse mutation assay. Consequently, the mutagenicity of TMAOH-capped FePt NPs was found to be weakly positive in the

Table 1. The numbers of total colonies including spontaneous revertant colonies that appeared on a plate treated with AET-capped FePt NPs

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	117 ± 6	130 ± 3	9 ± 3	9 ± 0	30 ± 1	40 ± 2	16 ± 0	29 ± 7	7 ± 0	10 ± 1
39.1	119 ± 3	123 ± 5	7 ± 1	7 ± 1	39 ± 4	35 ± 2	22 ± 2	25 ± 7	8 ± 3	12 ± 1
78.1	125 ± 5	124 ± 11	9 ± 1	8 ± 0	31 ± 6	37 ± 5	19 ± 4	22 ± 4	7 ± 1	11 ± 1
156	123 ± 5	119 ± 13	9 ± 2	8 ± 1	30 ± 1	35 ± 3	23 ± 4	31 ± 0	6 ± 0	8 ± 1
313	127 ± 9	120 ± 4	10 ± 1	7 ± 0	28 ± 1	40 ± 4	16 ± 1	24 ± 2	8 ± 1	10 ± 1
625	121 ± 7	121 ± 4	9 ± 3	9 ± 0	39 ± 1	30 ± 1	20 ± 1	22 ± 5	7 ± 1	12 ± 1
1250	117 ± 8	136 ± 8	13 ± 0	8 ± 3	38 ± 4	39 ± 1	18 ± 0	24 ± 5	6 ± 1	9 ± 1
2500	154 ± 16	129 ± 1	10 ± 3	12 ± 1	34 ± 3	34 ± 3	21 ± 4	29 ± 4	6 ± 1	10 ± 3
5000	150 ± 4	128 ± 4	13 ± 0	15 ± 2	40 ± 3	43 ± 4	19 ± 1	29 ± 4	8 ± 2	10 ± 0
Positive	950 ± 35	864 ± 30	434 ± 12	308 ± 16	632 ± 29	757 ± 82	557 ± 12	434 ± 7	311 ± 98	134 ± 12
	(0.01)	(1.0)	(0.5)	(2.0)	(2.0)	(10)	(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 and TA100 strains, ENNG for the WP2*uvrA*$^-$ strain, 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).

TA100 strain without S9 mix, though the mutagenicity of TMAOH itself was negative (Maenosono *et al.*, 2007). On the contrary, the mutagenicity of AET-capped FePt NPs was negative in the present study as mentioned above. The antimutagenicity of AET has been shown in combination with several chemical mutagens (Hartman and Shankel, 1990; Hoffmann *et al.*, 1999). Hoffmann *et al.* (1999) studied the protective effects of AET against the genotoxicity of β-propiolactone (β-PL) and bleomycin (BLM) (Hoffmann *et al.*, 1999). They found that two fundamentally different mechanisms of antimutagenicity: direct interception of an electrophilic mutagen (β-PL) by a nucleophilic antimutagen and depletion of molecular oxygen that is required for mutagenesis by BLM. In the case of TMAOH, hydroxide ions adsorb mainly on Fe sites on the FePt NP surfaces and stabilize NPs. In the case of AET, however, Pt sites on the NP surfaces are selectively covered with AET due to the strong Pt-thiol interaction (Fig. 2) (Tanaka and Maenosono, 2008). Pt is known to be an excellent catalyst for oxygen reduction. In addition, catalytic activity of FePt NPs for the preferential CO oxidation reaction has been reported to be higher as compared to that of Pt NPs, because of a noncompetitive dual site mechanism (Yin *et al.*, 2008). It means that platinum site in Pt^0 state acts as CO adsorption site and iron site in FeO_x or Fe^0 state as an O_2 dissociative-adsorption site enhances the surface reaction between the reactants on the neighboring sites. Hence, the results suggest that some catalytic effects of FePt NPs might be responsible for the mutagenicity of bare FePt NPs, and AET effectively block the mutagenic activity of FePt NPs via selective adsorption on Pt sites. Alternatively, AET molecules desorped from the surfaces of FePt NPs may exert the protective effects against the genotoxicity of bare FePt NPs.

In vitro chromosomal aberration test

The results of the *in vitro* chromosomal aberration test of AET-capped FePt NPs are shown in Table 2. In the 24-hr continuous treatment without S9 mix, FePt NPs caused significant increase in the frequencies of cells with chromosomal aberrations at the dose range of 600-800 μg/ml (6-27% of aberrant cells). Cytotoxicity of over 50% in the CHL/IU cells was observed at 800 μg/ml. In the short-term treatments with and without S9 mix, it was negative at doses up to 300 and 600 μg/ml, respectively. However, clear increases in the frequencies of cells with chromosomal aberrations were observed at 400 μg/ml (11% of aberrant cells) for +S9 mix, and at the dose range of 800-1,000 μg/ml (10-21% of aberrant cells) for −S9 mix. The majority of the aberrations were chromatid breaks and chromatid exchanges (Table 2). The results suggest that AET-capped FePt NP is clastogenic to cultured CHL/IU cells regardless of metabolic activation. However, normal aberration frequencies are seen at levels of cytotoxicity slightly less than 50% in all cases as shown in Table 2. It is widely accepted that chromosomal aberrations can be formed as an indirect consequence of high levels of cell killing (Kirkland, 1992; Hilliard *et al.*, 1998). Therefore, clastogenicity of AET-capped FePt NPs may be false-positive. At the present stage, a comparative discussion is difficult, because there is no report regarding the genotoxicity of FePt NPs to the best of our knowledge.

In conclusion, mutagenicity of AET-capped FePt NPs was found to be negative in the Ames test, while clastogenicity of AET-capped FePt NPs seemed to be false-positive in the *in vitro* chromosomal aberration test using CHL/IU cells. Further detailed *in vitro* genotoxicity tests, such as DNA adduct studies, are necessary to conclude that a positive aberration result is irrelevant.

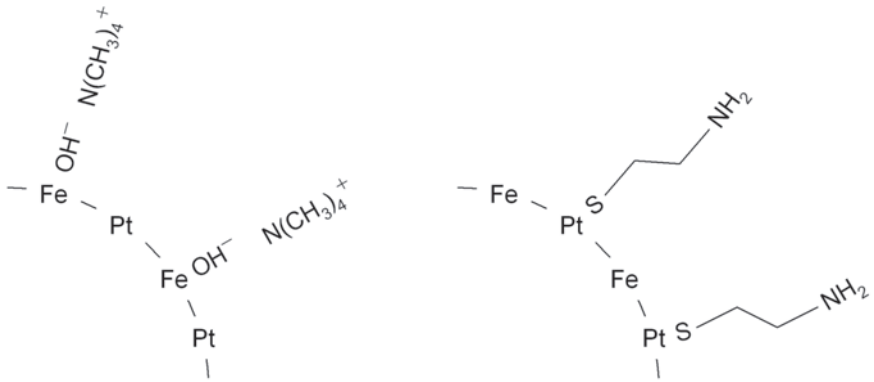

Fig. 2. Schematic illustrations of TMAOH (left) and AET (right) adsorption on the FePt NP surface.

Table 2. Chromosome analysis of Chinese hamster lung fibroblast cells treated with AET-capped FePt NPs

Compound	S9	Time (hr)	Dose (μg/ml)	CG (%)	Polyploid (%)	Judge	Frequency of cells with chromosomal aberrations (%)							
							ctb	cte	csb	cse	frg	gap	total	Judge
FePt	−	24-0	0	100	1	−	0	0	0	0	0	0	0	−
			200	113	NT		NT	NT	NT	NT	NT	NT	NT	
			300	81	NT		NT	NT	NT	NT	NT	NT	NT	
			400	78	0	−	0	0	0	0	0	0	0	−
			600	68	4	−	4	3	1	0	0	0	6	±
			800	42	2	−	20	23	2	0	0	0	27	+
			1,000	NT	NT		NT	NT	NT	NT	NT	NT	NT	
MMC			0.05	NT	0	−	25	25	0	0	0	0	35	+
FePt	−	6-18	0	100	0	−	0	0	0	0	0	0	0	−
			200	117	NT		NT	NT	NT	NT	NT	NT	NT	
			300	106	NT		NT	NT	NT	NT	NT	NT	NT	
			400	89	NT		NT	NT	NT	NT	NT	NT	NT	
			600	88	0	−	0	0	0	0	0	0	0	−
			800	59	1	−	9	8	1	0	0	0	10	+
			1,000	40	4	−	14	18	3	0	0	0	21	+
MMC			0.1	NT	0	−	27	28	0	0	0	0	38	+
FePt	+	6-18	0	100	0	−	0	0	0	0	0	0	0	−
			200	72	0	−	1	0	0	0	0	0	1	−
			300	50	0	−	0	0	0	0	0	0	0	−
			400	40	0	−	6	7	1	0	0	0	11	+
			600	NT	NT		NT	NT	NT	NT	NT	NT	NT	
			800	NT	NT		NT	NT	NT	NT	NT	NT	NT	
			1,000	NT	NT		NT	NT	NT	NT	NT	NT	NT	
BP			20	NT	0	−	44	78	0	0	0	0	78	+

Time = treatment time − recovery time. The negative control was injection solvent. The positive control used during −S9 mix was MMC. The positive control used during +S9 mix was BP. ctb, chromatid breaks; cte, chromatid exchanges; csb, chromosome breaks; cse, chromosome exchanges (including dicentric and ring chromosomes); frg, fragmentation; gap, chromatid and chromosome gaps; CG, cell growth; NT, not tested.

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