JAIST Repository

https://dspace.jaist.ac.jp/

Title	Evaluation of genotoxicity of amine-terminated water-dispersible FePt nanoparticles in the Ames test and in vitro chromosomal aberration test
Author(s)	Maenosono, Shinya; Yoshida, Rie; Saita, Soichiro
Citation	The Journal of Toxicological Sciences, 34(3): 349-354
Issue Date	2009
Туре	Journal Article
Text version	publisher
URL	http://hdl.handle.net/10119/9191
Rights	Copyright (C) 2009 日本トキシコロジー学会. Shinya Maenosono, Rie Yoshida, and Soichiro Saita, The Journal of Toxicological Sciences, 34(3), 2009, 349–354. http://dx.doi.org/10.2131/jts.34.349
Description	



Japan Advanced Institute of Science and Technology

Letter

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

Shinya Maenosono¹, Rie Yoshida¹ and Soichiro Saita²

School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Nomi, Ishikawa 923-1292, Japan

²Mitsubishi Chemical Group Science and Technology Research Center, Inc., 1000 Kamoshida-cho, Aoba-ku, Yokohama, Kanagawa 227-8502, Japan

(Received February 25, 2009; Accepted March 19, 2009)

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-

Correspondence: Shinya Maenosono (E-mail: shinya@jaist.ac.jp)

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 Fe₃(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 Xray 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



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

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.

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, MgCl₂, KCl, Dglucose-6-phosphate, β-NADPH, β-NADH, and sodium phosphate. Thus, 1 ml of S9 mix contained 0.1 ml of S9, 8 µmol of MgCl₂, 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 WP2uvrA- strain, sodium azide (NaN₃) 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 μ g/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 \ge 5% but < 10%, and positive (+) if \ge 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 μ g, 78.1 μ g, 156 μ g, 313 μ g, 625 μ g, 1,250 μ g, 2,500 μ g or 5,000 μ g 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 μ g/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 TMAOHcapped FePt NPs was found to be weakly positive in the

Dose (mg/plate)			Base-pair sul		Frameshift type						
	TA	100	TA1535		WP2	uvrA-	T	498	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)	

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

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, NaN₃ 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).

S. Maenosono et al.

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 Pt⁰ state acts as CO adsorption site and iron site in FeO_x or Fe⁰ state as an O₂ 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 24hr 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 μ g/ml (6-27% of aberrant cells). Cytotoxicity of over 50% in the CHL/IU cells was observed at 800 µg/ml. In the shortterm 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 falsepositive 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.

Compound S9	50	Time	Dose	CG	Polyploid	Indee	Frequency of cells with chromosomal aberrations (%)							
	(hr)	(µg/ml)	(%)	(%)	Judge	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	+

Evaluation of genotoxicity of amine-terminated FePt nanoparticles

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

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.

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. We also thank Mr. H. Saito from Mitsubishi Chemical Safety Institute Ltd. for the *in vitro* chromosomal aberration test. 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. S. Maenosono et al.

REFERENCES

- Dobson, J. (2006): Gene therapy progress and prospects: magnetic nanoparticle-based gene delivery. Gene, Ther., 13, 283-287.
- Gu, H., Ho, P.L., Tsang, K.W., Wang, L. and Xu, B. (2003): Using biofunctional magnetic nanoparticles to capture vancomycinresistant Enterococci and other gram-positive bacteria at ultralow concentration. J. Am. Chem. Soc., 125, 15702-15703.
- Hardman, R. (2006): A toxicologic review of quantum dots: toxicity depends on physicochemical and environmental factors. Environ. Health Perspect., 114, 165-172.
- Hartman, P.E. and Shankel, D.M. (1990): Antimutagens and anticarcinogens: A survey of putative interceptor molecules. Environ. Mol. Mutagen., 15, 145-182.
- Hilliard, C.A., Armstrong, M.J., Bradt, C.I., Hill, R.B., Greenwood, S.K. and Galloway, S.M. (1998): Chromosome aberrations *in vitro* related to cytotoxicity of nonmutagenic chemicals and metabolic poisons. Environ. Mol. Mutagen., **31**, 316-326.
- Hoffmann, G.R., Shorter, R.A., Quaranta, J.L. and McMaster, P.D. (1999): Two mechanisms of antimutagenicity of the aminothiols cysteamine and WR-1065 in *Saccharomyces cerevisiae*. Toxicol. in Vitro, **13**, 1-9.
- Kang, S., Shi, S., Nikles, D.E. and Harrell, J.W. (2008): Easy control of the size and composition of FePt nanoparticles with improved synthesis. J. Appl. Phys., **103**, 07D503.
- Kirkland, D.J. (1992): Chromosomal aberration tests in vitro: problems with protocol design and interpretation of results. Mutagenesis, 7, 95-106.
- Maenosono, S. and Saita, S. (2006): Theoretical assessment of FePt nanoparticles as heating elements for magnetic hyperthermia. IEEE Trans. Magn., 42, 1638-1642.
- Maenosono, S., Suzuki, T. and Saita, S. (2007): Mutagenicity of water-soluble FePt nanoparticles in Ames test. J. Toxicol. Sci., 32, 575-579.
- Miyake, Y., Wakata, A., Shimada, H., Morita, K. and Asaba, S., Eds. (2000): *Iyakuhin no tameno idendokusei siken Q&A*; Scientist Inc.: Tokyo
- Oberdörster, G., Sharp, Z., Atudorei, V., Elder, A., Gelein, R., Kreyling, W. and Cox, C. (2004): Translocation of inhaled ultrafine particles to the brain. Inhal. Toxicol., **16**, 437-445.
- Sun, S., Murray, C.B., Weller, D., Folks, L. and Moser, A. (2000): Monodisperse FePt nanoparticles and ferromagnetic FePt nanocrystal superlattices. Science, 287, 1989-1992.
- Sun, S. (2006): Recent advances in chemical synthesis, self-assembly, and applications of FePt nanoparticles. Adv. Mater., 18, 393-403.
- Tanaka, Y. and Maenosono, S. (2008): Amine-terminated water-dispersible FePt nanoparticles. J. Magn. Magn. Mater., 320, L121-L124.
- Wilcox, P., Naidoo, A., Wedd, D.J. and Gatehouse, D.G. (1990): Comparison of Salmonella typhimurium TA102 with Escherichia coli WP2 tester strains. Mutagenesis, 5, 285-291.
- Yahagi, T., Nagao, M., Seino, Y., Matsushima, T. and Sugimura, T. (1977): Mutagenicities of N-nitrosamines on salmonella. Mutat. Res., **48**, 121-129.
- Yin, J., Wang, J., Zhang, T. and Wang, X. (2008): Novel aluminasupported PtFe alloy nanoparticles for preferential oxidation of carbon monoxide in hydrogen. Catal. Lett., **125**, 76-82.
- Zhao, M., Beauregard, D.A., Loizou, L., Davletov, B. and Brindle, K.M. (2001): Non-invasive detection of apoptosis using magnetic resonance imaging and a targeted contrast agent. Nature Med., 7, 1241-1244.

354