TÜBİTAK

Turkish Journal of Biology

http://journals.tubitak.gov.tr/biology/

Research Article

Turk J Biol
(2014) 38: 283-288
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doi:10.3906/biy-1307-37

Effect of nanosized TiO_2 particles on the development of *Xenopus laevis* embryos

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Received: 15.07.2013 • **Accepted:** 13.12.2013 • **Published Online:** 28.03.2014 • **Printed:** 28.04.2014

Abstract: This paper reports the toxic properties of nano-TiO_2 on 2 different early life stages of *Xenopus laevis*. Synthesis of nano-TiO_2 particles was carried out by the hydrothermal method. Characterization of nanoparticles was performed using spectral techniques including X-ray diffraction, scanning electron microscopy, and a particle-size analyzer. Embryos at the 8th stage and tadpoles at the 46th stage were exposed to 7 concentrations of nano-TiO_2 in the range of 5 to 320 ppm. After 96 h of exposure, the mortality percentage of each exposure concentration was calculated and the activity of enzyme biomarkers acetylcholinesterase, carboxylesterase, glutathione S-transferase, glutathione reductase, lactate dehydrogenase, and aspartate aminotransferase were determined in living embryos and tadpoles. None of the tested concentrations of TiO_2 caused statistically significant mortality or malformation (only for the embryo test) as compared to the control groups. Furthermore, we did not observe any significant changes in enzyme activities in tadpole samples from the 46th stage, although some minor changes not related to the concentrations were observed in embryos.

Key words: TiO_2, hydrothermal process, *Xenopus laevis,* biomarker

1. Introduction

Nanotechnologies focus on the creation or manipulation of particles and materials of nanometric dimensions (from 1 to 100 nm). This particle size opens up a wide range of interesting applications including the development of drug and gene delivery systems. Gene delivery has become an increasingly important strategy for treating a variety of human diseases, including infections, genetic disorders, and tumors (Kubota et al., 1994; Kim et al., 2009; Pissuwan et al., 2011). The use of this technology is also gradually increasing in cosmetics, suntan lotions, paints, self-cleaning windows, stain-resistant clothing, antibacterial surfaces (Sayılkan et al., 2009), and photocatalysis (Sayılkan et al., 2007).

According to the Project on Emerging Nanotechnologies, the number of consumer products on the market containing nanoparticles or nanofibers has now exceeded 800 products and is still growing rapidly. According to The Nanotechnology Consumer Products Inventory, the most common materials mentioned in product descriptions were silver (390 products); titanium dioxide (182 products); carbon (82 products), which included fullerenes and nanotubes; zinc oxide (37 products), and finally cerium oxide (2 products) (Project on Emerging Nanotechnologies, 2013). The development of nanotechnologies and their widespread use are way ahead of the assessment of their impact on the environment, plants, animals, and humans; moreover, the currently available data are still contradictory (Andrievsky et al., 2005; Oberdorster et al., 2005). The sensitivity of the early life stages of amphibians to environmental pollution makes them good bioindicators to determine the effects of environmental chemicals. Amphibians are vulnerable to xenobiotic toxicity for the simple fact that they spend certain periods of their lives in both terrestrial and aquatic ecosystems (Pašková et al., 2011). *Xenopus laevis* is a well-known amphibian species that has adapted well to laboratory conditions (Prati et al., 2000). The embryos and tadpoles of *X. laevis* are important models that have been used in assessing the toxicity of compounds in embryonic development (Rizzo et al., 2007; Güngördü et al., 2013; Pekmezekmek et al., 2013).

Determination of changes in biochemical markers such as detoxification and metabolic enzyme as early warning signs of exposure to environmental pollutants might be used as a suitable tool for investigating sublethal

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toxic effects in amphibians (Venturino et al., 2003). Out of a variety of available biochemical markers, a set of enzymes including glutathione S-transferase (GST), glutathione reductase (GR), acetylcholinesterase (AChE), carboxylesterases (CaEs), lactate dehydrogenase (LDH), and aspartate aminotransferases (AST) activities were assessed as potential biomarkers that can be used to predict the toxic effects of short-term exposure of *X. laevis* embryos and tadpoles to TiO_2.

The aim of this study was to evaluate the toxicity of the TiO_2 on *X. laevis* tadpoles at different developmental stages in order to find out more about their toxic potential.

2. Materials and methods

2.1. Materials

Titanium tetraisopropoxide (TTIP) (Alpha; 97%) was used as precursor of titania. i-Propyl alcohol ($PrOH^i$), purchased from Riedel de Haen, was used as a solvent after drying over molecular sieves (Fluka, 3 Å XL8) for 1 day. The water used in the experiments was doubly distilled and deionized.

2.2. Preparation of nanosized TiO_2 particles and nanosols

The synthesis of nanosized particles was carried out as follows. First, TTIP was dissolved in i-propanol. After stirring for 5 min at ambient temperature, an i-propanol and hydrochloric acid mixture was added dropwise into an alkoxide solution at a rate of 1 mL/min. After stirring for 5 min, a water and i-propanol mixture was added to the solution dropwise at approximately the same rate. The mixture was stirred at ambient temperature for 10 min. The sol-solution obtained was then transferred to a stainless Teflon-lined autoclave and heated at 200 °C for 4 h. The mole ratios of $PrOH^i$/TTIP, H_2O/TTIP, and HCl/TTIP were 15, 2.62, and 0.2, respectively. The amount of TTIP was 14% by weight in all mixtures. The powders obtained by the hydrothermal process were isolated by centrifugation and dried in a vacuum sterilizer at 40 °C for 3 h. Eventually nanosized TiO_2 was obtained. Before examination of the toxicity for *X. laevis*, TiO_2 sols were prepared. For this purpose, certain amounts of TiO_2 were ultrasonically dispersed without addition of any dispersing agent. In this way, transparent sols were obtained.

2.3. Characterization

The major phase of the samples was determined by X-ray diffraction (XRD) patterns using a Rigaku Geigerflex Model D/Max-B diffractometer. Diffraction patterns were taken over the 2θ range of 0-70. For scanning electron microscopy (SEM), a LEO EVO 40 model microscope connected to a Rontek X-flash detector was used. Particle size was determined with the Malvern Zetasizer Nano Series. The XRD patterns of nano-TiO_2 particles, SEM microphotographs of nano-TiO_2, and size distribution of the TiO_2 dispersed in water (1%, w/w) are shown in Figures 1–3, respectively. We obtained quite similar nanoparticles as characterized by XRD patterns and SEM images. It was found that the size of the TiO_2 particulates was 4.78 nm with 96% by volume.

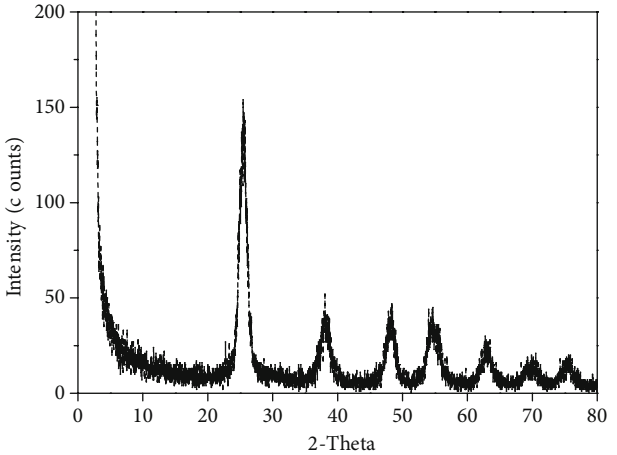

Figure 1. XRD of nano-TiO_2 particles.

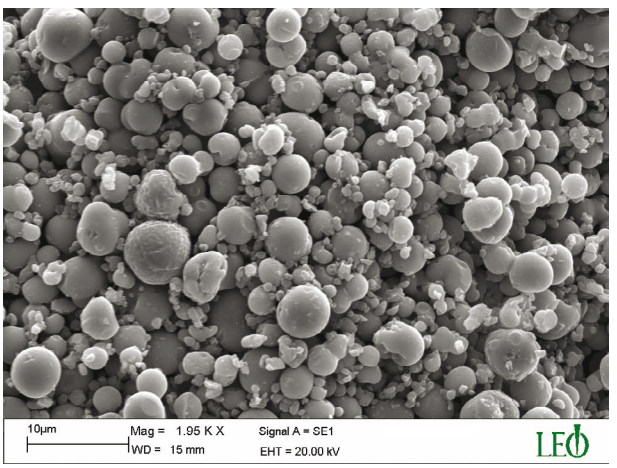

Figure 2. SEM image of nano-TiO_2.

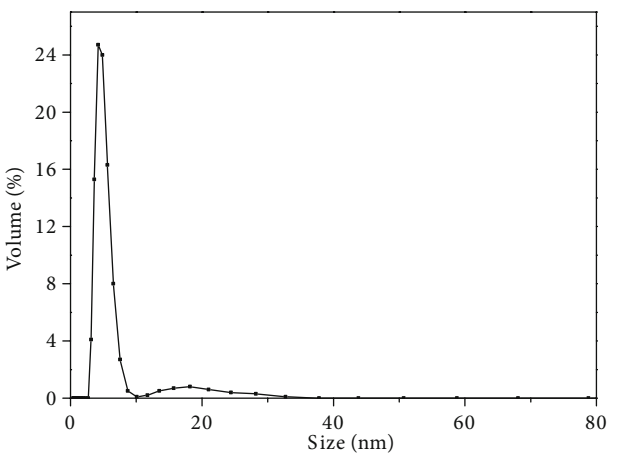

Figure 3. Size dispersion of nano-TiO_2.

2.4. Test organisms

Xenopus laevis embryos and tadpoles were used as test organisms. The breeding of adult *X. laevis* and the collection of embryos were performed in accordance with ASTM E1439-98 (ASTM, 1999). Fertilized eggs were selected and the embryos that proved to be developing healthily were maintained in well-aerated frog embryo teratogenesis assay-*Xenopus* (FETAX) solution. The FETAX solution was composed of 625 mg of NaCl, 96 mg of $NaHCO_3$, 30 mg of KCl, 15 mg of $CaCl_2$, 60 mg of $CaSO_4 \cdot 2H_2O$, and 75 mg of $MgSO_4$ per liter of distilled water. Average values for pH and conductivity of the FETAX solution were 7.4 ± 0.09 and, 1.42 ± 0.08 mS/cm, respectively. Embryos at the 8th stage and tadpoles at the 46th stage were used for FETAX and tadpole toxicity tests, respectively (Nieuwkoop and Faber, 1956).

2.5. Toxicity tests

For the FETAX and tadpole toxicity tests, 4 groups with 20 individuals (embryos or tadpoles) in each group (80 individuals in total) were randomly placed in petri and polycarbonate dishes, respectively, and were exposed to varying concentrations of TiO_2 for 96 h. TiO_2 stock and dilution were prepared in FETAX solution. The TiO_2 solution volumes were 25 mL and 50 mL for the FETAX and tadpole toxicity tests, respectively. The temperature of the laboratory was simultaneously stable, set at 23 ± 1 °C. The incidence of dead and living tadpoles was recorded for both tests after 96 h of exposure. Each embryo of the FETAX test was evaluated for malformations by using a dissecting microscope. During the following exposure, living embryos and tadpoles were collected and placed in a microcentrifuge vial. Each vial was chilled with ice and was stored at –80 °C until analyzed.

2.6. Biochemical assay

The embryos and tadpoles were thawed in an ice box, weighed, and homogenized at 1500 rpm for 30 s with a Teflon glass homogenizer with 10 strokes (model RZR-2021, Heidolph, Germany) along with ice-cold homogenization buffer. Four volumes of homogenization buffer [0.1 M K-phosphate buffer (pH 7.4)] with 0.15 M KCl, 1 mM ethylenediaminetetraacetic acid, and 1 mM dithiothreitol were used for each gram of tadpoles. The homogenates were centrifuged at 16,000 × *g* for 20 min at 4 °C, and the supernatants were transferred into clean microcentrifuge tubes. Immediately after the centrifugation procedure, enzyme activities in the postmitochondrial supernatants were determined.

All enzyme activities and total protein concentrations of samples were determined spectrophotometrically at appropriate wavelengths using a microplate reader (VersaMax, Molecular Devices Corp., USA) at 25 °C. Samples were assayed in triplicates. All enzyme activities were expressed as specific activity. AChE, CaE, GST, and GR activities were measured according to methods described in the literature with some modifications on microplate readers as described by Güngördü et al. (2013). AChE activity was measured by the method of Ellman et al. (1961). A modified version of Santhoshkumar and Shivanandappa's (1999) procedure concerning microplate readers was used to determine CaE activity. GST activity was measured in the postmitochondrial fraction of homogenates according to Habig et al. (1974). GR activity was measured using the method described by Stephensen et al. (2000), also with some modifications.

The LDH and AST assays were conducted using microplate reader commercial test kits (Biolabo, France) as provided by the manufacturer (Güngördü et al., 2013). The total protein concentration in the supernatant was measured using the Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as the standard (0-1.4 mg BSA/mL). Calculated protein values were used to further calculate the specific activity values for each enzyme tested.

2.7. Statistical analyses

Statistical analyses were performed using statistical software (SPSS Inc., USA). Differences of biochemical markers among the treatment groups were analyzed with the nonparametric Kruskal–Wallis test. If significant results were found, the Mann–Whitney U test was used to determine which treatment groups were significantly different from the controls. Differences were considered significant at $P < 0.05$. Furthermore, Dunnett's t-test was used to determine whether there were any statistically significant differences for mortality percentages caused by TiO_2 exposure in comparison with the control groups.

3. Results

None of the TiO_2 concentrations caused a statistically significant mortality percentage compared to controls in both the FETAX embryo (8th stage) and *X. laevis* tadpole (46th stage) applications (Table). The mortality percentages of control groups and the highest mortality percentages of TiO_2-treated groups were determined as 10% and 7.5% and as 17.5% and 7.5%, respectively, for both applications. On the other hand, according to the FETAX test results, no significant malformations were observed in TiO_2-treated embryos compared to the controls.

Throughout the study, toxic effects of TiO_2 on *X. laevis* tadpoles were also evaluated using biomarker enzymes (AChE, CAE, GST, GR, LDH, and AST). The AChE, GR, and LDH activities were different between the control groups of both applications ($P < 0.05$). The AChE activities of control groups were determined as 80.3 and 177.1 nmol min^{-1} mg $protein^{-1}$ for 8th stage embryos and 46th stage tadpoles, respectively. Biomarker enzyme activities in TiO_2-exposed tadpoles were not statistically significant

Table. The mortality percentage and biomarker enzyme activities of embryos and tadpoles of *Xenopus laevis* exposed to different concentrations of TiO_2.

Tadpoles	Conc. (ppm)	Mortality (%)	n	Biomarker enzymes†					
				AChE	GST	CaE	GR	LDH	AST
8th stage (FETAX)	0	10	4	80.3 ± 2.6	164.3 ± 4.3	136.1 ± 6.8	5.99 ± 0.67	248.1 ± 7.6	90.6 ± 6.6
	5	17.5	4	76.4 ± 3.8	158.1 ± 4.2	133.9 ± 7.2*	5.97 ± 0.78	204.3 ± 7.0	71.5 ± 4.1*
	10	12.5	4	90.9 ± 9.4	210.2 ± 22.3	113.2 ± 2.2*	8.20 ± 0.46	263.3 ± 24.3	81.1 ± 8.5
	20	8.75	4	78.8 ± 3.5	193.9 ± 14.4	107.0 ± 8.1*	7.42 ± 0.37	222.0 ± 13.8	66.8 ± 6.2
	40	13.75	4	90.2 ± 11.3	194.8 ± 10.8	113.6 ± 9.4	7.69 ± 0.57	269.1 ± 25.5	95.7 ± 11.9
	80	12.5	4	97.1 ± 9.4	198.4 ± 8.8	125.6 ± 8.0	7.56 ± 0.60	306.2 ± 20.1	115.9 ± 8.3
	160	13.75	4	114.1 ± 2.4	178.1 ± 20.8	108.5 ± 7.4*	6.97 ± 0.56	295.8 ± 28.0	101.4 ± 15.0
	320	15	4	100.3 ± 0.8	185.9 ± 26.9	106.9 ± 4.9*	5.98 ± 0.42	298.0 ± 33.1	110.1 ± 8.4
	640	10	4	-	-	-	-	-	-
	1280	16.25	4	-	-	-	-	-	-
46th stage	0	7.5	4	177.1 ± 13.6	153.4 ± 8.2	127.8 ± 15.3	11.03 ± 0.72	163.8 ± 22.7	61.9 ± 9.8
	5	1.25	4	173.8 ± 5.6	150.3 ± 5.1	143.0 ± 5.8	9.97 ± 1.17	152.9 ± 11.7	56.6 ± 5.5
	10	1.25	4	166.6 ± 5.5	174.9 ± 12.2	150.9 ± 5.0	10.36 ± 1.26	186.0 ± 18.4	67.1 ± 6.7
	20	0	4	156.3 ± 10.7	159.8 ± 4.9	137.5 ± 6.3	12.21 ± 1.52	166.4 ± 7.9	60.5 ± 5.7
	40	0	4	189.5 ± 16.2	172.3 ± 11.4	148.7 ± 5.0	11.37 ± 0.39	187.9 ± 15.5	65.0 ± 6.3
	80	6.25	4	184.7 ± 8.3	176.6 ± 12.1	144.2 ± 8.1	11.03 ± 0.68	179.2 ± 12.2	63.0 ± 5.9
	160	6.25	4	220.3 ± 15.4	159.0 ± 5.7	140.2 ± 9.4	10.91 ± 0.70	157.4 ± 6.4	52.0 ± 5.8
	320	7.5	4	197.5 ± 4.2	174.7 ± 9.6	142.0 ± 1.4	12.55 ± 1.35	164.2 ± 10.6	46.0 ± 4.1

All enzymes activities are expressed as nmol min^{-1} mg $protein^{-1}$ ± standard error. †: Enzyme activities were determined in living tadpoles after 96 h of TiO_2 exposure. *: $P < 0.05$, statistical significance compared with control.

compared with that in the control group (Table). On the other hand, although not directly related to dose, all biomarker enzymes were changed by one or more concentrations of TiO_2 at a statistically significant level in the 8th stage tadpole application. Applications of 160 and 320 ppm caused an increase in AChE activity, while the same concentrations caused a significant inhibition of CaE.

4. Discussion

Due to high amounts of metals in the environment as a result of human activities, numerous scientific studies have evaluated the effects of metals. On the other hand, there have been many studies on biological applications of TiO_2 in recent years, but there are a relatively small number of studies investigating toxic effects of nanometals on aquatic organisms (Yeo and Kang, 2006).

In the present study we investigated the toxicity of TiO_2 solutions at different concentrations on 2 different early life stages of *X. laevis*. According to the data obtained from this study, the concentrations of TiO_2 tested did not cause significant mortality rates or malformation. Similar to our study, a previous study showed that concentrations of up to 1000 ppm TiO_2 did not cause mortality or significant malformation in *X. laevis* embryos (Nations et al., 2011). In the other study, 96 and 48 h LC_{50} values of TiO_2 were determined as more than 1000 mg/L for a fish species, *Pimephales promelas* (Hall et al., 2009)

Furthermore, no statistically significant changes in enzyme activities of tadpoles were observed throughout the tests. On the other hand, exposure to high concentrations of TiO_2 (160 and 320 ppm) caused increases in AChE activity and inhibitions in CaE activity in embryos. Increases in AChE activity were shown previously in *X. laevis* embryos

and in a fish species, *Sparus aurata*, after copper exposure and also in mussel after lead and cadmium exposures (Romani et al., 2003; Bainy et al., 2006; Güngördü et al., 2010). One of the possibilities for the increase of AChE activity by metals may be due to de novo synthesis of this enzyme as a response to an initial inhibition (Bainy et al., 2006). Furthermore, it was shown in pesticide-exposed fish that AChE might be protected from inhibition stoichiometrically by CaE inhibition (Küster, 2005).

Although the embryos of *X. laevis* were relatively more susceptible to TiO_2 exposure than tadpoles, the concentrations of TiO_2 tested did not cause significant mortality or malformation neither in *X. laevis* embryos nor in tadpoles.

Acknowledgment
The authors are grateful for the financial support of İnönü University, Turkey (BAPB-2011/31).

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