Vibrational Spectroscopy 56 (2011) 146–153

Contents lists available at ScienceDirect

Vibrational Spectroscopy

journal homepage: www.elsevier.com/locate/vibspec

Raman spectroscopic investigation on the microenvironment of the liver tissues of Zebrafish (*Danio rerio*) due to titanium dioxide exposure

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ARTICLE INFO

Article history:
Received 2 June 2010
Received in revised form 5 January 2011
Accepted 26 January 2011
Available online 2 February 2011

Keywords:
Zebrafish
Liver
Titanium dioxide
Biochemical constituents
Microenvironments

ABSTRACT

Nano titanium dioxide ($nTiO_2$), generally considered to be toxicologically inert, is manufactured in large quantities and extensively applied in consumer products. The small size and large surface area endow them with an active group or intrinsic toxicity. Advances in instrumentation are making Raman spectroscopy the tool of choice for an increasing number of (bio) chemical applications. One of the great advantages of this technique is its ability to provide information on the concentration, structure and interaction of biochemical molecules in their microenvironments within intact cells and tissues, non-destructively. Zebrafish (*Danio rerio*), one of the most important vertebrate model organisms used in developmental biology, are increasingly used in biomedical research, particularly as a model of human disease. In the present work, an attempt is made to study the effect of titanium dioxide, both nano and bulk, on the microenvironment of the liver tissues of Zebrafish using FT-Raman spectroscopy. The results of the present study suggest that TiO_2 exposure demonstrate a marked influence on the microenvironments of the liver tissues of Zebrafish. A shift to a higher wavenumber and an increase in the intensity of the band at $\sim$1087 cm^{-1} in the TiO_2 exposed tissues suggest that some of the conformational changes resulting from the alkali recovery process takes place due to TiO_2 exposure. The decreased intensity ratio (I_{3220}/I_{3400}) observed in the titanium-exposed tissues suggests a decreased water domain size, which could be interpreted in terms of weaker hydrogen-bonded molecular species of water in the TiO_2 exposed tissues. The observed shift of COO^- bands to higher frequencies shows the disruption of salt bridges as a result of a change in the oppositely charged partners and due to the enhanced random coil conformation. The variation in the intensity ratio of the tyrosyl doublet (I_{858}/I_{825}) indicates variation in the hydrogen bonding of the phenolic hydroxyl group due to TiO_2 exposure. The results further suggest that the microenvironments are greatly altered due to titanium nano exposure when compared to titanium bulk. In conclusion, the results indicate that FT-Raman spectroscopy might be a useful tool for rapid assessment of nano particle biological interactions.

1. Introduction

With the rapid development of nanotechnology, the potential health hazards and environmental impacts of manufactured nanomaterials (MNMs) are receiving increasing attention. The first reported study on the developmental toxicity of metal oxides nanoparticles released into aquatic environment by Zhu et al. [1] reveals that some nanoscale metal oxide particles might exert detrimental effects to the aquatic ecosystem health. The pioneer study of Adams et al. [2] showed that the photosensitive nanomaterials were harmful to varying degrees, increasing with particle concentration. Titanium dioxide is a noncombustible and odorless white powder, frequently used as a white pigment for a wide range of paints, paper, plastics, ceramics, and the like. Nano titanium dioxide ($nTiO_2$), generally considered to be toxicologically inert, is manufactured in large quantities and extensively applied in consumer products such as sunscreen and cosmetics [3]. It is used widely because of its high stability, anticorrosion and photocatalysis [4]. TiO_2 nanoparticles have a bigger surface area compared with the materials of conventional size. The small size and large surface area endow them with an active group or intrinsic toxicity. Their physicochemical characteristics are different from those of conventional size TiO_2 because of the small-size effects, surface effect, quantum-size effect, and macroscopic quantum tunneling effect of TiO_2 nanoparticles. Studies on acute toxicity of TiO_2 nanoparticles in mammals indicate that they evoke inflammatory response and histopathological changes [5]. Bioaccumulation studies [6] confirmed that titanium was mainly accumulated in the liver tissue. With the increasing development of nanotechnology, more and more nanoparticles are entering into the environment. Studies

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doi:10.1016/j.vibspec.2011.01.005

[7,8] show that the major characteristics of TiO_2 nanoparticles are closely associated with their biological effects. The ultrafine size of TiO_2 nanoparticles enables them to pass through cell membranes and nuclear membranes, and they can affect cell ultrastructure and damage the cell membrane [9,10].

Raman spectroscopy is a non-destructive technique that provides information about the molecular structure of the investigated sample. Advances in instrumentation are making Raman spectroscopy the tool of choice for an increasing number of (bio) chemical applications. One of the great advantages of this technique is its ability to provide information on the concentration, structure and interaction of biochemical molecules in their microenvironments within intact cells and tissues, non-destructively, and without homogenization, extraction, or the use of dyes, labels, or other contrast enhancing agents [11]. The positions and relative intensities of the various spectral bands can be used to probe primary, secondary, tertiary and quaternary structures of large biological molecules.

A popular aquarium species, Zebrafish, *Danio rerio* (Hamilton) is one of the most important vertebrate model organisms used in developmental biology for many years. They are increasingly important in biomedical research [12], particularly as a model of human disease [13,14] and for the screening of therapeutic drugs [15]. Its strength as a model organism is that, as a vertebrate, it is more comparable to humans than invertebrate model species, while being more tractable to genetic and embryological manipulation than mammalian model species such as mice, in which such procedures are both more complicated and costly. The Zebrafish has now become a prominent research model, in large part because of its tolerance of a wide range of environmental conditions. Hence, in the present work an attempt is made to study the effect of titanium dioxide, both nano and bulk, on the microenvironment of the liver tissues of Zebrafish using FT-Raman spectroscopy.

2. Materials and methods

2.1. Test species

The tropical freshwater adult Zebrafish (*D. rerio*) of length 4 ± 0.2 cm and weight 5 ± 0.2 g were procured from Redline Farm House Pvt. Ltd. at Kolathure, Kancheepuram District, Tamil Nadu, India. The collected fish were transported to the laboratory in an oxygen pack. The fish were first treated with 1% potassium permanganate solution for 15 min to avoid any infection and were then maintained in glass aquaria of size 30 cm × 60 cm × 40 cm in the laboratory for 2 weeks prior to experimentation. For the entire duration of the experiment, the fish were fed with tropical fish food (Taiyo Pet products Pvt. Ltd., Chennai, India).

2.2. Test chemicals

Powder form of ultra fine titanium dioxide nanoparticles was obtained from Sigma–Aldrich Company, Bangalore, India and was the titanium (IV) oxide type (manufacturer's information); crystal structure was anatase T_iO_2; purity was at least 99.7% and an average particle size was <20 nm with an average surface area of $200 \pm 20\ m^2\ g^{-1}$.

Powder form of bulk titanium dioxide was obtained from Nice Chemicals Pvt. Ltd., Cochin, India and was of anatase structure, purity was at least 99.7% and average particle size was above 1000 ± 20 nm.

2.3. Particle characterization

The morphology of the samples was characterized using high-resolution transmission electron microscopy (HRTEM, JEOL model JEM 3010), installed at Sophisticated Analytical Instrumentation Facility (SAIF), IIT Madras, India. The samples for TEM observation were prepared by dispersing the products in milli Q water with an ultrasonic bath for 15 min, and then placing a few drops of the resulting suspension onto a copper grid.

X-ray diffraction analysis was carried out using the computer controlled X-ray Diffractometer (X'Pert PRO-PANalytical, Philips) installed at Central Instrumentation Facility (CIF), Pondicherry University, Puducherry, India.

2.4. Titanium dioxide nanoparticles stock solution and dosing

The LC_{50} values for TiO_2 NPs and TiO_2 bulk were determined by using Litchfield and Wilcoxon method [16] and were found to be 30 ppm and 300 ppm, respectively. Stock solutions of dispersed TiO_2 NPs were prepared by sonication after considering the recommendations of the manufacturer and the findings of Matthews [17]. A stock solution of 10 ppm $nTiO_2$ was prepared by dispersing the nanoparticles in ultrapure water (Millipore, ion free and unbuffered) with sonication for 6 h in a bath-type sonicator (BANDELIN Sonopuls HD 2070, BERLIN), and subsequently for a further 30 min sonication immediately prior to dosing each day. In the same way, 100 ppm of TiO_2 bulk stock solution was also prepared. Chemical analysis of stock solutions revealed no metal impurities. Dispersion was confirmed by transmission electron microscopy. The dispersion was very good at the final working concentrations (10 ppm of $nTiO_2$) and the measured particle size was close to the manufacturer's information. In order to achieve working concentrations of 100 ppm of bulk and 10 ppm of $nTiO_2$ in the fish tanks, each tank was dosed with 100 or 10 ml of the stock solution, respectively.

2.5. Experimental study

Fish were exposed in triplicate, each containing 20 fish, to one of the following treatments for 14 days: control (freshwater only), 10 ppm of titanium dioxide nanoparticles and 100 ppm of titanium dioxide bulk material. These concentrations were selected after conducting preliminary experiments. The aeration in the tank dispersed each dose around the tank within seconds in all experiments. The test media was renewed every day to maintain the exposure concentration.

The physico-chemical parameters such as pH, total alkalinity, total hardness, calcium, magnesium and DO were measured by standard methods [18] and maintained at optimum level (7.0–7.2, 210–220 mg/L, 290–296 mg/L as $CaCO_3$, 88–90 mg/L, 19–22 mg/L and 7.07–7.24 mg/L). Photoperiod was 12 h light:12 h dark.

At the end of the experimental period, the fish were sacrificed, liver organs were separated and stored at −80 °C until sample preparation for FT-Raman spectroscopic studies. No mortality was observed during the 14-day exposure period in any group.

2.6. Sample preparation

The liver tissues were dried in a lyophilizer (VIRTIS 6KBEL85) for 12 h to remove the water content in the samples. The samples were then ground in an agate mortar and pestle in order to obtain liver powder. The liver powder (5 mg) was mixed with completely dried potassium bromide (100 mg) in a mortar and redried to remove all traces of remaining water. The mixture was then subjected to a pressure of 5 tones for 5 min in an evacuated die to produce a clear transparent KBr disc of 13 mm diameter and 1 mm thickness for use in Raman analysis. In the present work, dried tissue samples were used to record the spectrum. This method was extensively used by number of workers [19–21] to study homogeneous tissue samples. In the drying process, free and unbound water was removed from