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The effect of titanium dioxide exposure on the thermal properties of Zebrafish (*Danio rerio*) bones

K. S. Pramod · V. Vijayasundaram · N. Krishnakumar · PL. RM. Palaniappan

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Abstract This article presents the changes in the thermal properties of the control and titanium dioxide (TiO_2), both nano and bulk exposed Zebrafish bones by using thermo analytical techniques. The result shows that the mass loss due to the thermal decomposition occurs in three distinct steps due to loss of water, organic and inorganic materials. The titanium dioxide exposed bones present a different thermal behaviour compared to the control bones. The residue masses are found to be increased due to titanium dioxide exposure. In particular, nano titanium dioxide exposure increases the residue mass level significantly (three fold) when compared to titanium dioxide bulk exposure. These thermal characteristics can be used as a qualitative method to check the metal oxide intoxication in biological samples.

Keywords Bone · TG-DSC-DTA · Thermal analysis · TiO_2 · Zebrafish (*Danio rerio*)

Introduction

Nanotechnology has now become an important industry, playing a significant role in the generation of new products and applications through the development of nanoparticles (NPs). Due to their specific physicochemical properties which may differ from the bulk substances, NPs provide novel prospects of commercial and scientific applications [1]. At the same time, NPs are found to be more toxic than larger particles of the same substance because of their larger surface area, enhanced chemical reactivity, and easier penetration of cells. With the growing commercialization of NPs, concerns about the exposure of NPs to humans and the environment have been increasing [2]. The NPs may be released into freshwater systems as a result of their commercial usage and environmental cleanup of waste in industry and medicine. It is a well-known fact that most of the industrial waste and all urban water sewage end up in waterways. Hence, it is inevitable that industrial nanoscale products and by-products enter the aquatic environment. A number of studies have shown the potential impact of NPs on a range of aquatic organisms and their toxicity can be related to dissolution, surface properties or size [3, 4]. Among all nanoparticles, titanium dioxide (TiO_2) nanoparticles are of biggest ecotoxicological concern due to the rapid increase of anthropogenic input of nano-TiO_2 into the environment. Titanium dioxide (TiO_2), a widely used mineral oxide in the cosmetics, pharmaceutical and paint industries, is considered to be physiologically inert and poses little risk to humans. However, when TiO_2 is made at the nanoscale, its biological and environmental effects deserve our emerging attention.

Fish constitute an important source of protein because it provides a healthy, low cholesterol source of protein and other nutrients. At the same time, levels of contaminants in fish are of considerable interest because of potential effects on the fish themselves or the organisms that consume them, including people. In polluted areas, exposure of fish to xenobiotics leads to interactions between these chemicals and biological systems, which lead to biochemical disturbances. Most of the metals in organisms accumulate in bones and can be liberated under some pathological

K. S. Pramod · N. Krishnakumar · PL. RM. Palaniappan (✉)
Department of Physics, Annamalai University,
Annamalai Nagar 608 002, Tamil Nadu, India
e-mail: plrmpl@hotmail.com

V. Vijayasundaram
Department of Physics, Adhiparasakthi Engineering College,
Melmaruvathur 603 319, Tamil Nadu, India

conditions [5]. Bones are complex in structure and are sensitive to environmental factors. It is a composite material consisting of ~10% water, 30% organic material (mainly collagen), and 60% inorganic material (predominantly carbonated hydroxyapatite) by weight [6]. The relative amounts of these constituents are variable depending on age, diet and health status. The earlier studies [7, 8] have shown that bone tissue is an endogenic metal source reflecting the total metal content in an organism. In addition, those studies indicate that the bone content of metals is mostly from environmental pollution.

Thermal analysis (TA) is a well-established set of techniques for obtaining qualitative and quantitative information about the effects of various heat treatments on materials of all kinds, including new chemical compounds, plastics, ceramics, alloys, construction materials, minerals, foods, and medicines [9]. Thermal methods have been used for the characterization of bone in several studies, having been applied to the characterization of bone from various species, the dating of archaeological and forensic remains and to the characterisation of both the organic and inorganic phases for the development of synthetic analogues [6]. As bones contain both inorganic and organic components, thermal methods lend themselves to the examination of both the inorganic and organic components of bone [10]. Thermogravimetry (TG) is one of the oldest thermal analytical procedures. In these techniques, the changes in the physical or chemical properties of the sample are measured as a function of temperature in the scanning mode or as a function of time in the isothermal mode [11]. This technique involves monitoring the mass loss of the sample in a chosen atmosphere, usually nitrogen or air, as a function of temperature. The usefulness of thermogravimetry (TG) for analyzing complex systems is greatly enhanced by the introduction of the ability to record simultaneously the first derivative of the mass loss. Due to the sensitivity of thermal analysis, thermogravimetry (TG) and differential scanning calorimetry (DSC) have been selected for this work. TG is inherently quantitative, and therefore an extremely powerful thermal technique, but gives no direct chemical information. The ability to analyze the volatile products during a mass loss is of great value. TG has been used extensively for the characterization of bones in a number of studies [12–17]. The ability of TG to generate fundamental quantitative data has led to its widespread use in every field of science and technology.

Differential scanning calorimetric examination allows demonstrating the thermal consequences of local as well as global conformational changes in tissue elements. DSC monitors the thermal response of a sample to temperature changes, or isothermally. The same temperature program is applied to a sample and a reference pan, and the difference in heat flow to each pan is measured. By observing the difference in heat flow between the sample and reference, differential scanning calorimeters are able to measure the amount of energy absorbed or released during such transitions. The transitions appear as endothermic peaks if heat is absorbed or as exothermic peaks if heat is given out. Foregoing examinations have demonstrated that DSC is a useful and well-applicable method for the investigation of biological systems [18] including human tissues [19] and medical problems [20]. This method is particularly appropriate to the study of collagen based tissues and materials like bone, because of an unusually large endotherm related to denaturation of type I collagen. To see whether there are differences in thermal behaviour between the control and titanium dioxide exposed fish bones, TG and DSC measurements were performed in this study. The aim of this paper is to study the effect of titanium dioxide, both nano and bulk, exposure on the thermal properties of Zebrafish (*Danio rerio*) bones using thermo analytical techniques in order to monitor the transformation process suffered by the bones. In the current investigation, Zebrafish bones are selected because they are rich in calcium, which is an essential mineral for normal body function. Zebrafish (*Danio rerio*) is a widely used model species for developmental biology, genetics, behavioural ecology and ecotoxicology [21]. Zebrafish has also been proven to be a powerful experimental system in elucidating complex biological processes. Many features of this tropical freshwater fish are recognized as advantageous for studying vertebrate development and eventually contribute to the success of Zebrafish as a model organism.

Materials and methods

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).

Test chemicals

Powder form of ultra fine nano titanium dioxide was obtained from Sigma-Aldrich Company, Bangalore, India

and was the titanium (IV) oxide type; crystal structure was anatase TiO_2; purity was at least 99.7% and an average particle size was < 20 nm with an average surface area of 200 ± 20 m^2 g^{-1}. Powder form of bulk titanium dioxide was obtained from Nice Chemicals Pvt Ltd. Cochin, India; purity was at least 99.7% and average particle size was above 1000 ± 20 nm.

Lethality studies

The 96 h LC_{50} value for TiO_2-NPs and TiO_2 bulk was determined by using Litchfield and Wilcoxon [22] method and was found to be 30 and 300 ppm, respectively. A sublethal level of TiO_2 (one-third of the LC_{50} value) was used in this study. The acclimated fish were stocked in glass aquaria equipped with continuous air supply. Stock solutions of TiO_2-NPs were prepared by sonication after considering the recommendations of the manufacturer and the findings of Matthews [23]. A stock solution of 10 ppm TiO_2-NPs 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 TiO_2-NPs) and the measured particle size was close to the manufacturer's information. In order to achieve working concentrations of 100 ppm of bulk TiO_2 and 10 ppm TiO_2-NPs in the fish tanks, each tank was dosed, respectively, with 100 or 10 mL of the stock solution.

Experimental study

Fish were exposed in triplicate, each containing 10 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 experiment was designed to allow for acute toxicity sub-lethal physiological effects over the exposure period rather than sub-lethal physiological effects. The exposure time of 14 days was chosen to reflect this dosimetry and enable biochemical responses to the exposure. The aeration in the tank dispersed each dose around the tank within seconds in all experiments. The test media was renewed every day at 8 am in order to maintain the exposure concentration. The physico-chemical parameters such as pH, total alkalinity, total hardness, calcium, magnesium, and DO were measured according to APHA [24] 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, fish were dissected and bones from each set of experimental fish were removed and used immediately for the thermal analysis. No mortality was observed during the 14 day exposure period in any group.

Sample preparation

The fish bone specimens were cleaned with a scalpel to remove any residues from the exterior of the specimens, washed with physiologic solution and treated in acetone to remove lipid residues. Then the specimens were dried in an air oven for 3 h to remove adsorbed water. Then the bones were powdered in an agate mortar and pestle in order to obtain bone powder.

Thermal analysis

In this study, the thermal analysis was carried out using a simultaneous thermogravimetry (TG) unit. The experiments were conducted in an air atmosphere using a simultaneous TG-DSC-DTA unit from TA Instruments, Model SDT Q600, available at the Central Electro Chemical Research Institute (CECRI), Karaikudi, Tamil Nadu, India. Platinum crucibles were used to hold the bone powdered samples. The bone samples were heated from ambient temperature to 1200 °C at a heating rate of 10 °C min^{-1} in an air atmosphere and mass loss in samples was measured. Calorimetric measurements were also performed using the same instrument. The peak temperature and enthalpy were determined numerically from thermograms for each exothermic process. The peak temperature was calculated as the temperature at minimum value of calorimetric signal within the exotherm. The enthalpy was determined from the area between exotherm and a baseline which was constructed by extrapolating the scan beyond the exotherm. Origin 8.0 did the data treatment after ASCII conversion.

Statistical analysis

All data were expressed as mean ± standard deviation. SPSS for Windows, Version 11.5, (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The differences between the corresponding values between the control and exposed groups were analyzed using analysis of variance (ANOVA) followed by Student's t test. A probability level (p value) of less than 0.05 is regarded as statistically significant.

Results and discussion

The TG–DTG–DTA curves are indicative of a complex thermal degradation mechanism which is specific to each species under study. The TG data usually show mass losses at particular temperatures that may be correlated with the organic and inorganic phases in the sample. The first mass loss step usually occurs from ~50 °C up to 220 °C and corresponds to water desorption. In the second step, the mass loss observed from 220 °C up to 600 °C represents the combustion of the organic components to produce carbon dioxide, water and organic fragments. In the third step, observed in the range 600–1200 °C, the mass loss is associated with the release of carbon dioxide from the carbonated hydroxyapatite, if the decomposition is carried out in an air atmosphere [25].

Thermogravimetric analysis

To our best knowledge the effect of titanium dioxide, both nano and bulk, on the thermal properties of Zebrafish (*D. rerio*) bones, has not been studied and published in literature before. The observed TG curves for the control, TiO_2 bulk and $n$$TiO_2$ exposed *D. rerio* bones are presented in Fig. 1. These curves show the mass loss due to the thermal decomposition in an air atmosphere which occurs in three distinct regions and these mass losses are presented in Table 1. Region 1 is observed from 50 to 220, 232 and 236 °C for the control, TiO_2 bulk and $n$$TiO_2$ exposed bones, with a peak at 78, 83 and 78 °C, respectively. The most important changes in this region are in some physical properties and little mass loss. The mass loss in this region is characteristic of released hydroscopic water. The mass loss in this region is 12.37, 9.45 and 9.19% for the control, TiO_2 bulk, and $n$$TiO_2$ exposed bones, respectively.

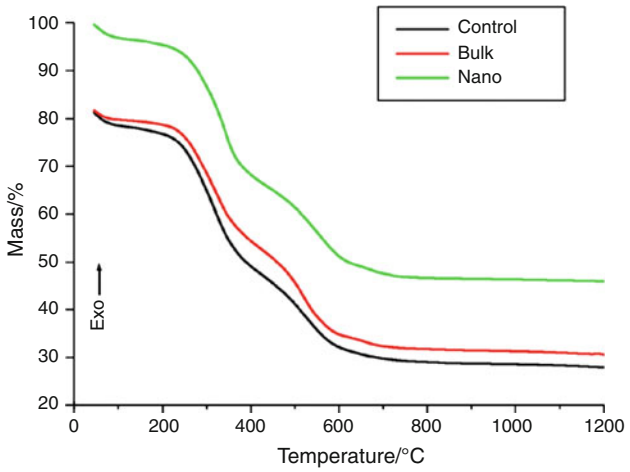

Fig. 1 The average TG curves for the decomposition of the control, TiO_2 bulk and $n$$TiO_2$ exposed Zebrafish bones

Table 1 Total mass losses in different regions for the control, $n$$TiO_2$ and TiO_2 bulk exposed Zebrafish (*Danio rerio*) bones

Samples	Percentage of mass loss			Residue mass percentage at 1200 °C
	Region 1	Region 2	Region 3	
Control	12.37 ± 0.48	75.59 ± 3.36	7.48 ± 0.31	4.56 ± 0.12
TiO_2 bulk exposed	9.45 ± 0.33	74.41 ± 2.46	6.93 ± 0.29	9.21 ± 0.38
$n$$TiO_2$ exposed	9.19 ± 0.23	55.31 ± 2.24	8.17 ± 0.38	27.33 ± 1.12

Water and organic contents are calculated as a percentage of the starting mass; each value is the mean ± SD of ten individual observations. The degree of significance was denoted as: $p < 0.05$

The mass loss in the Region 2 is very fast and significant and characterized by three steps due to decomposition process of organic material. First decomposition of organic material can be attributed to the phosphate decomposition with a mass loss of 27.29%, taken between 220 and 328 °C. The second decomposition of organic material due to lipid decomposition between 328 and 502 °C with 31.94% of mass loss takes place. Between 502 and 605 °C, there is a third decomposition with 16.36% of mass loss, probably due to the mineral salts' decomposition [26]. In an oxidizing atmosphere, the organic phase is expected to be removed up to 605 °C. The mass loss between 220 and 605 °C is 75.59, 74.41, and 55.31% of the original mass for the control, TiO_2 bulk and $n$$TiO_2$ exposed bones, respectively.

The final stage of mass loss is observed between 605 and 1200 °C. The mass loss in this region was measured to be 7.48% based on the original mass, giving a total mass loss of 95.44% for the control. The total mass loss was 90.79% for TiO_2 bulk exposed bones and 72.67% for $n$$TiO_2$ exposed bones. The mass loss in this Region 3 agrees with the earlier reported values [27, 28] and can be attributed to the decomposition of the inorganic phases, notably the removal of carbon dioxide from the carbonated apatite as has been observed by Sohar et al. [12]. In this study, the mass loss is found to be decreased due to TiO_2 exposure when compared to the control. This might be due to the degeneration of organic matrix due to TiO_2 exposure. Similar results were observed by Utech et al. [29] in pathologic human bone samples.

Differential scanning calorimetry

The calorimetric study is based on the assumption that the macromolecules of biological systems are in complex interactions with their environment. Any change in the external chemical-physical variables (e.g., temperature) results in characteristic changes of the system, which can

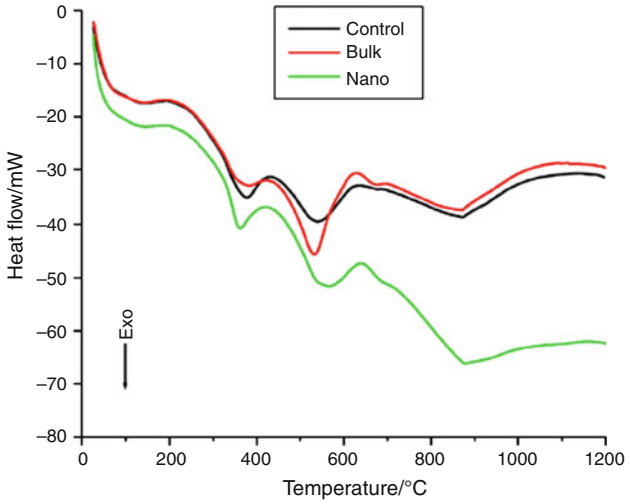

Fig. 2 The average DSC curves for the control, TiO_2 bulk and $n$$TiO_2$ exposed Zebrafish bones

be detected by calorimetry. If a biological structure undergoes a change for any reason, its thermodynamic characteristics will change, and its calorimetric graph will deviate from the original [30]. To see whether there are differences in thermal behaviour between the control, TiO_2 bulk and $n$$TiO_2$ exposed *D. rerio* bones, DSC measurements were performed.

Figure 2 shows the DSC curves for the control, TiO_2 bulk and $n$$TiO_2$ exposed *D. rerio* bones. In this study, the DSC curves show three well-defined exothermic peaks due to three effects of mass loss. Region 1 is associated with the evolution of water. The observed mass loss may be accounted for by the onset of decomposition processes which evolve water as well as organic species. Loss of water content is presented in the DSC curves (Fig. 2) for all the three samples, starting from 50 to 138, 140, and 130 °C, respectively, for the control, TiO_2 bulk and $n$$TiO_2$ exposed fish bones. The underlying reason could be that the thermal heat capacity of any biologic system is basically dependent on the amount of water tied [18].

Region 2 is characterized by a decomposition process of the organic material identified with the sharp exothermic peaks in the DSC curves at 374 and 540; 366 and 537; and 360 and 542 °C for the control, TiO_2 bulk and $n$$TiO_2$ bone samples, respectively. Most probably the peak in the DSC curves in this region is caused by the dissociation of $CaCO_3$ [31]. Similar results were observed by Utech et al. [29] in healthy and pathologic human bones.

The thermal decomposition of the samples is observed throughout Region 3 as the curves are not observed to reach zero even at 1200 °C. In this region only 7.48% of mass loss is observed in the control bones, whereas 6.93 and 8.17% of mass loss is observed in TiO_2 bulk and $n$$TiO_2$ exposed bones. This mass loss may be due to the evolution of organic products as suggested by Onishi et al. [25]. In the TiO_2 bulk and $n$$TiO_2$-exposed bone samples, an increase of the slope is observed in the region 800–1200 °C with the peak centred around 874 °C for both TiO_2 bulk and $n$$TiO_2$ exposed bones indicating a broad exothermic band. Although there is likely to be some decomposition of the mineral phase in this region, the mass increase appears to be predominantly associated with the organic products or may be due to the oxidation of free titanium cation as suggested by Rincón et al. [32].

As seen from the TG graphs, the maximum loss is observed in the second region for all the samples. In general, maximum mass loss is observed in the control bones and minimum in the TiO_2 exposed bones. This might be due to TiO_2 exposure. Further, the mass loss is minimum in $n$$TiO_2$ when compared to TiO_2 bulk exposed bones. This suggests that the $n$$TiO_2$ accumulated more in the bones when compared to bulk TiO_2 exposure. The exothermic peaks and their calorimetric enthalpy values for the control, TiO_2 bulk and $n$$TiO_2$ exposed bones are presented in Table 2. From the Figure and Table, it is observed that the peak at 540 °C gives an enthalpy of 1040 J g^{-1} for the control, the peak at 537 °C gives an enthalpy of 1698 J g^{-1} for the TiO_2 bulk exposed bones and the peak at 543 °C gives the calorimetric enthalpy of 958.4 J g^{-1} for $n$$TiO_2$ exposed bones, respectively. Greatest change in the enthalpy was observed in the TiO_2 bulk exposed bones. Therefore, denaturation caused by heating was largest in the TiO_2 bulk exposed bones when compared to TiO_2 nano. Consequently these samples required the largest amount of energy for decomposition. The DSC results clearly suggest that definitive differences exist between the thermal

Table 2 The calorimetric enthalpy changes observed in the control, $n$$TiO_2$ and TiO_2 bulk exposed Zebrafish (*Danio rerio*) bones

Samples	Peak 1/°C	ΔH/J g^{-1}	Peak 2/°C	ΔH/J g^{-1}	Peak 3/°C	ΔH/J g^{-1}	Peak 4/°C	ΔH/J g^{-1}	Peak 5/°C	ΔH/J g^{-1}
Control	138.32	81.19 ± 4.01	374.43	657.60 ± 30.54	540.15	1040 ± 35	680	16.04 ± 0.70	874.38	880.70 ± 40.03
TiO_2 bulk exposed	140.02	91.40 ± 4.15	366.23	367.10 ± 16.22	537.03	1698 ± 49	671.71	47.97 ± 2.27	874.35	569.90 ± 25.45
$n$$TiO_2$ exposed	129.72	101.40 ± 4.92	359.90	400.50 ± 11.25	542.73	958.40 ± 47	682.87	25.46 ± 1.23	874.35	621.70 ± 30.81

Each value is the mean ± SD of ten individual observations. The degree of significance was denoted as $p < 0.05$

characteristics of the control, TiO_2 bulk and $n TiO_2$ exposed *D. rerio* bones. The significant change in the calorimetric enthalpy in the TiO_2 exposed bones might be due to the change in the amount of collagen, and in the amount of secondary bindings between the collagen fibres [20].

The thermal decomposition of bone mineral appears to depend on the organic content of the bone. In this study, the residue masses for the control, TiO_2 bulk and $n TiO_2$ exposed bones are 4.56, 9.21 and 27.33% respectively. The increased residue content in the TiO_2 (both bulk and nano) exposed bones might be due to the accumulation of TiO_2. Further, in the $n TiO_2$ exposed bones, the residue masses increased nearly threefold (27.33%) when compared to the TiO_2 bulk exposed bones (9.21%). The significant increase in the residue mass level due to $n TiO_2$ exposure could be related to the smaller size of the particles, their increased reactivity as a result of greater surface area per particle, or the greater number of particles in a dose. This is further evidenced by the ICP-AES studies which show that the amounts of TiO_2 concentration in the bones of *D. rerio* are 5.3, 17.98 and 21.52 ppm, respectively, for the control, TiO_2 bulk and $n TiO_2$ exposed bones. Hence, this study clearly shows that the nano particle accumulation changes the thermal behaviour of fish bones. This is evidenced by the drastic change noticed in the calorimetric enthalpy values when compared to bulk material.

Conclusions

The results of this study demonstrate the potential of TG as a technique for evaluating the metal intoxication in bone specimens. The TiO_2 (both bulk and nano) exposed bones present a different thermal behaviour when compared to the control bones. The significant increase in the residue mass level due to $n TiO_2$ exposure could be related to the smaller size of the particles, their increased reactivity as a result of greater surface area per particle, or the greater number of particles in a dose. In addition, the calorimetric enthalpy values have changed drastically due to $n TiO_2$ exposure, when compared to control and TiO_2 bulk exposed bones. These thermal characteristics can be advantageously used as a qualitative method to check the metal oxide intoxication in biological samples.

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