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RESEARCH ARTICLE

Biosynthesis of Iron oxide nanoparticles and its haematological effects on fresh water fish *Oreochromis mossambicus*

S. Karthikeyeni[1], T. Siva Vijayakumar[1], S. Vasanth[1], Arul Ganesh[1], M. Manimegalai[2] and P. Subramanian[1*]
[1]Dept. of Animal Science, Bharathidasan University, Tiruchirappalli-620024, TN, India
[2]Dept. of Zoology, Seethalakshmi Ramasamy College, Tiruchirappalli-620024, TN, India
subbus1952@gmail.com*; +91 9787639657

Abstract

Iron oxide nanoparticles were synthesized biologically using potato as starch template ($FeSo_4.7H_2O$ as substrate). The X-ray diffraction (XRD) and Scanning Electron Microscope (SEM) analysis revealed that the particles were spherical in shape and the size ranges from 29-40 nm. Toxicity tests were performed to investigate the possible harmful effects on fresh water fish *Oreochromis mossambicus* exposed to as synthesized α-Fe_2O_3 NPs at exponential concentrations (0.5, 5 and 50 µg/mL). The significant changes in haematological (RBC, WBC, Hb, HCT) and biochemical parameters (SGOT, SGPT) were observed after 96 h in treated groups than control, which may be a consequence of iron oxide NPs in the blood cells of fish.

Keywords: Iron oxide nanoparticles, toxicity, fresh water fish, *Oreochromis mossambicus*, SGOP, SGPT.

Introduction

Nanoscience is the study of materials on the nanoscale level approximately between 1 and 100 nm (Rotello, 2003) and deals with manipulation of formation of two and three-dimensional assemblies of molecular scale building blocks into well-defined nanostructures or nanomaterials (Rosi and Mirkin, 2005). Metal oxide nanoparticles are extensively used in a considerable number of applications in food, material, chemical and biological sciences (Aitken *et al.,* 2006). It is well known that bulk materials based on TiO_2, SiO_2, aluminium and iron oxides have been massively produced for many years. More recently, nanoparticulate versions of these metal oxides have been manufactured and introduced in commercial products such as cosmetics and sunscreens (TiO_2, Fe_2O_3 and ZnO) (Nowack and Bucheli, 2007), fillers in dental fillings (SiO_2) (Balamurugan *et al.,* 2006), in catalysis (TiO_2) (Aitken *et al.,* 2006), and as fuel additives (CeO_2) (Laosiripojana *et al.,* 2005). However, due to the increasing interest of these nanomaterials to be used as potential devices for biomedical applications, water soluble iron oxide NPs is an active area of research.

Iron is necessitated by most of living organisms because it is required for many to execute metabolic processes including oxygen transport, drug metabolism, steroid synthesis, DNA synthesis, ATP production and electron transport (Crichton, 1991). However, the use of iron in biological systems is associated with two problems: low solubility of free metal ions and generation of toxic oxidants. Nowadays, biomaterials are widely utilized as stabilizing agents in experimental procedures to save the environment which may easily recyclable or degradable. Starch from natural sources has been advantageous to stabilize iron oxide nanoparticles (Barsby *et al.,* 2001).

Hence, our study aimed at exploiting a common biomaterial starch, a carbohydrate polymer as a template for synthesizing Fe nanoparticles. Due to their importance in medicine and nutrition starch from natural source was chosen for the synthesis. Copolymer templates were used to host chemical reactions which exhibit advantageous property of avoiding nanoparticle clustering and also providing stable frameworks against chemical degradation (Dresco *et al.,* 1999). Stabilisation with capping agents does not only help to protect the iron oxide NPs against degradation, it can also be used for the introduction of specific functional groups into the NP surface, such as catalytically active species, various drugs and specific binding sites (Dyal *et al.,* 2003; Willner and Katz, 2003). These nanoparticles have been reported to possess potential application in the cancer diagnosis as magnetic resonance imaging agents. Although nanomaterials are currently being widely used in modern technology, there is a serious lack of information concerning the human health and environmental implications of manufactured nanomaterials. A limited number of studies have been done with fish as a model organism. Kashiwada *et al*. (2006) showed that latex nanoparticle was taken up by the eggs of fish *Oryzias latipes* and subsequently the nanoparticles were accumulated in gills, intestine, brain, testis, liver and blood of hatched adult fish. Smith *et al.* (2007) reported the pathophysiological effects by the single walled carbon nanotubes to rainbow trout (*Oncorhynchus mykiss*). Although, much has been studied about the haematological parameters of fish (Blaxhall and Daisy, 1973; Walencik and Witeska, 2007), to our knowledge this is the first study to determine the health status of *O. mossambicus* when exposed to iron oxide nanoparticles.

Serum GOT and GPT (Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT)) catalyze the transfer of the amino group from an alpha amino acid forming a new amino acid and a new keto acid. Many disturbances in liver functions were produced due to changes in the concentrations of the Transaminase (Maxine and Binjamin, 1985; Marie, 1994; Shalaey, 2000). Hence, GOT and GPT is considered as a predominant biomarker for hepato toxicity studies. The present study was focalise to estimate the potential effects of iron oxide nanoparticles on the experimental fish (*O. mossambicus*) based on the determination of the haematological changes in a dosage dependent manner.

Materials and methods

Chemicals and reagents: All the chemicals used in this experiment were of analytical grade. Ferrous sulfate, heptahydrate ($FeSO_4.7H_2O$, ≥ 99.5%), RBC and WBC diluting fluids were purchased from HiMedia (Mumbai, India) and used without further purification.

Synthesis of iron nanoparticles: Iron oxide nanoparticle was prepared by using 25 mL of potato extract as starch template with 2 g of $FeSO_4.7H_2O$ and stirred for 30 min. The template iron mixed solution (highly viscous) was treated at 60°C (heating hike rate of 5°C/min) and maintained at that temperature for 4 h, after which it was cooled to room temperature at a rate of 10°C/min. Reddish brown particles were obtained which was further characterized using XRD and SEM.

Experimental protocol: Oreochromis mossambicus of average length of 9-10 cm and weighing 15-20 g were segregated into 3 groups each with 10 fish per group. Fish belonging to both the sexes were used. Concentrations of 0.5, 5, 50 µg/mL of starch (rice-porridge) assisted synthesized iron oxide nanoparticle was diluted by dechlorinated water and administered orally to fishes. All experimental fish were fed daily. Blood was drawn from both control and a treated fish on 24, 48, 72, and 96 h. Care was taken to avoid stress during sampling.

Preparation of samples: Blood was drawn from control and experiment group fish by cardiac puncture using plastic disposable syringe fitted with 26 gauge needle. The syringe and needle were prechilled and moisturized with heparin (Heparin sodium salt, Sisco research laboratories pvt., Ltd, Mumbai, India). Blood was transferred into small vials, which was previously rinsed with heparin. The whole blood was used for the estimation of hemoglobin, erythrocytes and leucocytes count. The blood was allowed to clot for 30 min at ambient temperature (19-24°C) and the upper layer of fluid was collected as serum and used for further analysis.

Analytical procedure: To assess the hematological profile of the control and treated fish, hemoglobin, hematocrit (HCT), RBC and WBC counts were measured from the whole blood of *O. mossambicus*. Erythrocytes (RBC) and total leucocytes (WBC) were counted with a Neubauer hemacytometer with respective diluting fluids. The mean cellular volume (MCV), mean cell haemoglobin (MCH) and mean cellular haemoglobin concentration (MCHC) were also calculated by standard formulas (Kang *et al.,* 2005). Hemoglobin and hematocrit (packed cell volume) content of the blood was estimated by Sahli's acid method using Plane Haemometer (HiMedia, Mumbai). Erythrocytes and leucocytes were counted by the method of Rusia and Sood (1992) using haemocytometer. The blood biochemical parameters like Serum Glutamic Oxaloacetic Transaminase (SGOT) and Serum Glutamic Pyruvic Transaminase (SGPT) were analysed using assay kits, purchased from Biosystems, Spain. Procedure was followed by instructions in the assay kit and absorbance (OD) was taken at 340 nm.

Results and discussion

In this work, starch from the natural sources (rice porridge) has been employed as template to synthesize iron nanoparticles and its haematological effects on *O. mossambicus* was also studied. The characterization of the crystallite nature of iron oxide was carried out by using XRD analysis, which is essential for identification of the product (Schwertmann and Cornell, 1991; Cornell and Schwertmann, 2003). The crystal structure and phase composition of the prepared samples was achieved by investigating the XRD patterns (Fig. 1). The crystallite size was calculated using the Debye-Scherrer formula, $D = 0.9\lambda/\beta Cos\theta$, where D is the particle size, λ the wavelength of the X-ray used, β and θ are the half-width of X-ray diffraction lines and half diffraction angle of 2θ. The diffraction peaks coincided well with the value of JCPDS card 84-0306, which could be well indexed to the pure hexagonal phase of hematite ((012), (104), (110), (113), (024), (116), (214), and (300)). The crystallite size was found to range between 19 and 33 nm and confirmed as α-Fe_2O_3 of iron oxide by JCPDS card no. 84-0306. The XRD structural analysis of the as prepared sample shows pure hematite phase of iron oxide (Zhang *et al.*, 2011).

Fig. 1. X-ray diffraction pattern of as prepared iron oxide nanoparticles.

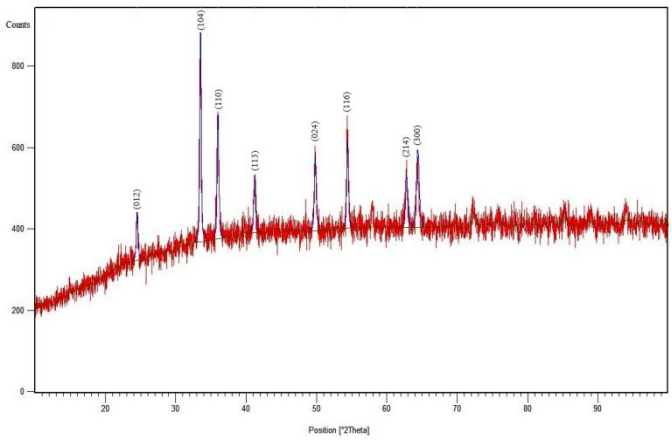
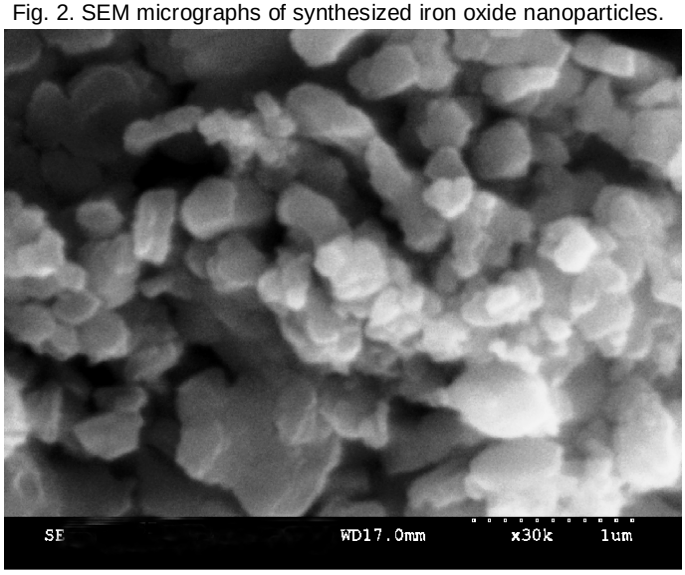


Table 1. Over time alterations of the hematological parameters of control and α-Fe_2O_3 NPs treated *O. mossambicus*.

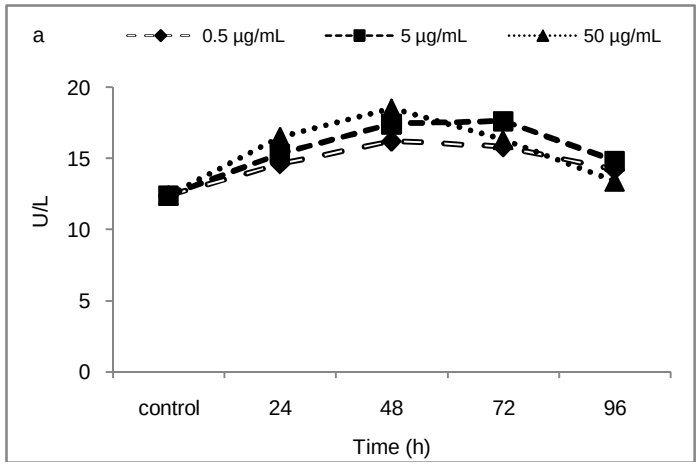
Conc. of α-Fe_2O_3NPs	Hours	RBC (10^6 mm-3)	WBC (10^3 mm-3)	Hb (g dL^{-1})	HCT (%)	MCV (μ^3)	MCH (pico. g)	MCHC (g/dL)
	Control	**0.25**	**85.75**	**5.2**	**12.6**	**504.00**	**208.00**	**41.27**
	24	0.39	52.75	7.5	23.5	602.56	192.31	31.92
0.5 µg/mL	48	0.31	42.10	6.0	20.6	664.51	193.55	29.13
	72	0.21	80.25	5.0	11.2	535.71	238.10	44.44
	96	**0.20**	**73.00**	**4.2**	**11.7**	**587.50**	**240.00**	**40.85**
	Control	**0.25**	**85.75**	**5.2**	**12.6**	**504.00**	**208.00**	**41.27**
	24	0.32	66.00	6.5	23.5	734.37	244.44	27.66
5 µg/mL	48	0.46	50.50	7.2	26.4	573.91	203.13	27.27
	72	0.18	88.25	4.4	11.0	611.11	217.39	40.00
	96	**0.23**	**81.00**	**5.0**	**11.9**	**517.31**	**156.52**	**42.02**
	Control	**0.25**	**85.75**	**5.2**	**12.6**	**504.00**	**208.00**	**41.27**
	24	0.53	55.75	7.2	31.5	594.34	135.85	22.86
50 µg/mL	48	0.35	54.25	6.2	26.0	742.86	178.57	24.04
	72	0.17	87.00	4.6	10.9	641.17	270.58	42.20
	96	**0.20**	**78.00**	**4.8**	**11.2**	**510.00**	**210.00**	**41.18**

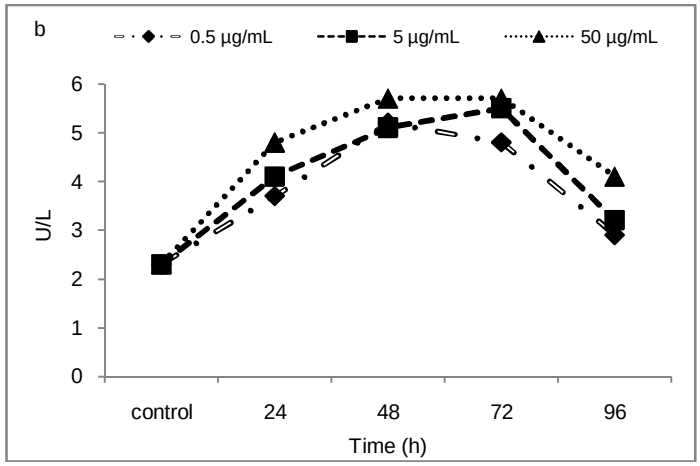
Fig. 2. SEM micrographs of synthesized iron oxide nanoparticles.



The SEM micrograph shows a large quantity of formation of nanoscale iron oxide particles of hexagonal nanoclusters in shape (Fig. 2) with a diameter of about 40-50 nm. The templates provided only the binding sites for the Fe(II) centers, which were then converted during the heat treatment to iron oxides. Khan *et al.* (2011) also reported the similar structure for α-Fe_2O_3 NPs. Hematological assays may provide an index of the physiological status of fish, synthesis and analysis of the figures obtained for the individual nanoparticles may provide valuable information on the specific response or the range and nature of the pathological process (Rehulka, 2002). As to the blood parameters, the α-Fe_2O_3 NPs treated *O. mossambicus* had lower erythrocytes and hemoglobin levels from the 48 h to the end of the experimental period for all concentrations. Erythrocytes were 0.25 x 10^6 mm-3 and hemoglobin levels were 5.2 for the healthy fish (control). Hematocrit values at 24 and 48 h were found significantly higher than the other times (Table 1).

Fig. 3. Over time (a) GOT and (b) GPT enzyme activities of α-Fe_2O_3 NPs treated *O. mossambicus* at different concentrations.





Fluctuating leukocyte counts observed in our study. Leukocyte values obtained on 72 h of the experiment were significantly higher in 0.5 µg/mL concentration whereas for 5 and 50 µg/mL on 96 h.

Within the red blood cell indices, higher MCV, MCH and lower MCHC values were reported after 96 h. Similarly, RBC and Hb were found to be significantly lower as were the MCV and MCH higher while MCHC showed no significant differences between diseased and healthy fish (Tripathi *et al*., 2005).

Determination of blood serum transaminase enzymes Glutamic-acid-oxylo acetic-acid-transaminase-GOT) and glutmaic-acid-pyruvic-acid-transaminase-GPT is a routine method in human hepatic health diagnostics (Jeney *et al.,* 1992). GOT is also known as Aspartate Aminotransferase (AST) and GPT as Alanine Aminotransferase (ALT) are cytoplasmic hepatocellular enzymes whose increase in blood is highly indicative for liver damage (Bell, 1968; Gaudet *et al.,* 1975; Bouk *et al.,* 1978). The activity of GOT and GPT enzyme level was increased with the increased concentration of α-Fe_2O_3 NPs till 48 h which fell to near normal levels after 96 h (Fig. 3a and 3b). Among the chosen time intervals, 48 h samples showed significant elevated levels of the enzymes. Thus, it is evidenced from the results that α-Fe_2O_3 NPs can damage the liver of *O. mossambicus* at 50 µg/mL with an acute period of exposure. Similar depurative study was done by Amutha and Subramanian (2009) on *O. mossambicus* exposed to ZnO NPs at different concentrations (70 to 100 ppm).

Conclusion

Nanotechnologies hold great promise for reducing the production of wastes, reducing industrial contamination and improving the efficiency of energy production and use. However, the production, use and disposal of manufactured NPs will inevitably lead to discharges to air, soils and aquatic systems. Uptake of nanoparticles into aquatic organisms, including fish, was shown previously (Kashiwada, 2006). The fact that the size of the particles itself can be a factor in direct toxicity and pathology is extremely important, and biodegradability may be a further significant factor in governing harmful biological effects (Brown *et al.,* 2001; Hoet *et al.,* 2004; Howard, 2004). Compared to other methodologies, use of natural polymers as templates for synthesis of iron oxide nanoparticles seems beneficial to the environment even when the synthesis was carried out at high temperatures. The particles of iron oxide obtained are of nano-dimensions with average size in the range of 140-200 nm. From the depurative results of hematological and biochemical assays it was evident that the biosynthesized (rice porridge as template) α-Fe_2O_3 was less toxic to *O. mossambicus* up to 50 µg/mL. But elevated level of GOT and GPT enzymes till 48 h showed that the fish may get hepatic tissue damages when exposed to higher doses of iron NPs. Hence, more studies in the field of nanotoxicolgy were needed at the moment to protect the aquatic environment from the hidden danger.

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