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IN VITRO EFFECT OF IRON OXIDE NANOPARTICLES ON XANTHINE OXIDASE ACTIVITY

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Abstract

Recently, the boom of metal oxide nanoparticles synthesis and its biomedical applications have led to emphasize on the possible impact of these nanoparticles on bio-macromolecules. In this study, we studied the impact of green Fe_2O_3NPs and chemically synthesized (Commercial), on xanthine oxidase (XO) activity by addition of CuO-NPs in aqueous suspension (1–100µg/ ml at an interval of 10µg) in the reaction mixture of its spectroscopic assay. The XO assay was also performed *in vivo* using fresh human serum in place of XO. The XO activity was decreased, as the concentration of Fe_2O_3NPs increased. Km value of XO was increased upon addition of both types of Fe_2O_3NPs indicating the lower affinity of enzyme towards the substrate in presence of Fe_2O_3NPs. Our results provide insight into the interaction of Fe_2O_3NPs with enzyme, which can be important for further applicability of nanoparticles in biomedical applications.

Key words : Iron oxide nanoparticles and xanthine oxidase activity.

Introduction

Today nanotechnology is one of the most active research areas of science. It is a branch of science which deals with particles less than 100 nm in size which is known as nanoparticles (NPs). NPs have a wide range of applications in various fields. Most of the nano-products produced on an industrial scale are used in skin care products that use NPs to deliver vitamins deeper into the skin, sunscreens that use NPs to block UV rays without leaving white residue on the skin, toothpastes, textiles, sports equipment, sanitary ware coatings, food products, medicine and diagnostics (Ball, 2001). Despite the wide applications of nanomaterials, there has been a serious lack of information concerning the impact of NPs on human health and the environment. In general, NPs have the ability to interact with whole physiological surrounding once when they enter human body. In order to optimize the beneficial effects of NPs on living organisms, it is essential to understand the fundamental interactions of it with biological systems (Komatsu *et al.*, 2008). In most of the cases, first molecules they interact with are proteins (Ren *et al.*, 2011). All enzymes are proteins therefore, the effects of NPs on enzymes and their activity are important to study. Enzyme kinetics is a field of biochemistry concerned with the quantitative measurement of the rates of enzyme-catalyzed reactions and the systematic study of factors that affect the reaction rates. A complete, balanced set of enzyme activities is of fundamental importance for maintaining homeostasis of body (Randle 1963). Therefore, understanding of enzyme kinetics is important to study (Kennelly and Rodwel, 2009). Effect of NPs on enzyme kinetics can be studied by incubating the enzyme with NPs and determining its enzyme activity to find out that whether the NPs were inhibiting or activating the enzyme activity (Lynch and Dawson, 2008). Till date no data is available to study the effect of NPs on xanthine oxidase. Xanthine oxidase (XO) is a metallo-protein that catalyzes the formation of xanthine which can further catalyze the oxidation of xanthine to uric acid according to the following equations:

$$\text{Hypoxanthine} + O_2 \rightarrow \text{Xanthine} + H_2O_2$$

$$\text{Xanthine} + O_2 \rightarrow \text{Uric acid} + H_2O$$

XO is widely distributed, occurring in milk, small intestine, kidney and liver. XO is of major medical interest as a target of many drugs against several diseases in

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humans, such as gout, hyperuricaemia and chronic heart failure (Stiefel, 1993). Accumulation of uric acid can result in hyperuricaemia, leading to arithritis and gout (Enemark and Young, 1993). The effects of NPs on key mediators of biological functions such as enzymes have been studied very less (Thoenes *et al.*, 1994). Although information about toxicity of metal oxide NPs continues to increase, a significant knowledge gap still exists on a complete toxicological profile of metal oxide NPs proposed for future use in medical applications. Without the data, risk assessment or regulation for safety of the materials shall suffer immeasurably.

The present study describes the comparative effect of green and chemically synthesized (commercial) Fe_2O_3NPs (which is synthesized and characterized in our paper Hooda *et al.*, 2019) on activity/kinetics of XO *in-vitro* and vivo (in human serum). The study could also help to further evaluate the long-term impact of NPs on XO.

Materials and Methods

Chemicals and reagents

Xanthine, xanthine oxidase (XO) from bovine serum, 4-aminophenazone, ethylene diaminetetra–aceticacid (EDTA), diethyldithiocarbamate (DDC), N-ethylmaleimide (NEM), sodium molybdate, sodium thiocyanate, NaCl, $MnCl_2$, $CaCl_2$, $ZnSO_4$, $MgSO_4$, $FeSO_4$, $CuSO_4$, riboflavin, sodium nitrate, FAD, FMN, NAD, sodium azide, ampicillin and peroxidase from horseradish were from SRL Mumbai. Other chemicals were of analytical reagent grade. Distilled water (DW) was used throughout the study.

Assay of native/free XO

Assay of XO was accomplished according to Pundir *et al*., (1998).

XO assay *in vivo*

In the assay mixture, XO was replaced by 1.0 ml fresh human serum. The remaining procedure of the assay was similar to as given in the assay of native/free XO.

Effect of Fe_2O_3NPs on XO *in-vitro*

To study the effect of Fe_2O_3NPs (both green and chemical) on XO activity, in the assay mixture 0.1 ml of buffer was replaced by the same amount of aqueous suspension of Fe_2O_3NPs solution (1–100µg/ ml at interval of 10µg). pH of the reaction buffer was maintained at 8.2 and 9.0, when incubated with green and chemical Fe_2O_3NPs respectively. The remaining procedure of the assay was similar to as given in the assay of native/free XO.

Effect of Fe_2O_3NPs on XO *in vivo*

To study the effect of Fe_2O_3NPs (both green and chemical) on human serum XO activity, in the assay mixture 0.1 ml of buffer was replaced by the same amount of aqueous suspension of Fe_2O_3NPs solution (1–100µg/ ml at interval of 10µg). pH of the reaction buffer was maintained at 8.2 and 9.0, when incubated with green and commercial Fe_2O_3NPs respectively. The remaining procedure of the assay was similar to as given for the assay of XO in vitro.

Effects of Fe_2O_3NPs on kinetic parameters of XO

The kinetic parameters of XO were studied in the presence of both green and chemical Fe_2O_3NPs.

To determine optimum pH of XO, the pH of reaction mixture was changed from pH 3.0 to 10.0 at 0.5 pH interval, all at a final concentration of 0.05 M. To study the optimum incubation temperature of XO, the reaction mixture was incubated at different temperatures ranging from 25 to 80 ^{0}C at a 5 ^{0}C interval. To study the effect of substrate concentration, the concentration of xanthine was varied between 0.1 to 50 mM, Km for xanthine was measured from the effect of substrate on XO as follow: Km = [S] at 1/2Vmax for comparison purpose.

Effect of metal chelators

To study of the effect of metal chelators, following chelators such as sodium azide, DDC, NEM, EDTA, sodium molybdate, sodium nitrate and sodium thiocyanate, were added in the reaction mixture individually containing green / chemical Fe_2O_3NPs at a final concentration of 1.0 mM.

Effect of metals

To study the effect of metals on XO, the following metals salts $CaCl_2$, $FeSO_4$, $MnCl_2$, $ZnSO_4$, $CuSO_4$, NaCl, and $MgSO_4$ were added in the reaction mixture individually at a final conc. of (1.0 mM), in the presence of both Fe_2O_3NPs.

Effect of coenzymes

To study the effect of coenzymes, FMN, FAD, NAD and riboflavin were added in reaction mixture (0.1 mM) previous to start the reaction, individually in presence of Fe_2O_3NPs.

Results and Discussion

Effect of Fe_2O_3NPs on XO activity both *in-vitro* and *in vivo*

When chemically and green Fe_2O_3-NPs (1-100 µg/ ml) were added in the reaction mixture of XO, the enzyme activity was inhibited in both the cases as shown in Fig. 1. The XO activity without addition of Fe_2O_3-NPs was

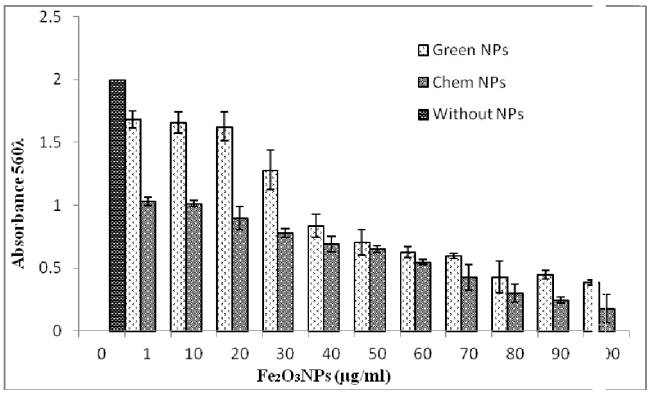

Fig. 1: Effect of Fe_2O_3-NPs on the activity of XO invitro.

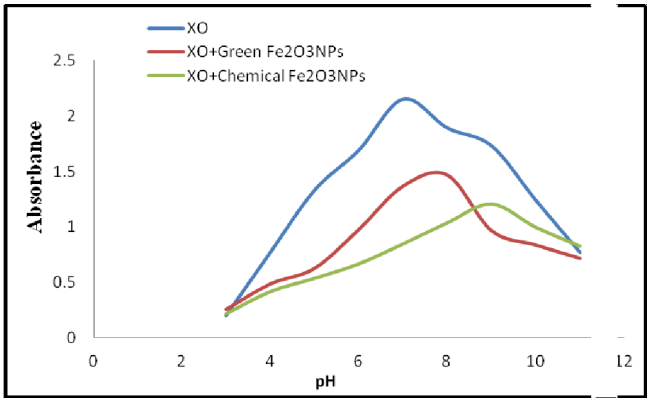

Fig. 3: Effect of pH on activity of xanthine oxidase (XO) in-vitro, in presence of green and chemical Fe_2O_3-NPs.

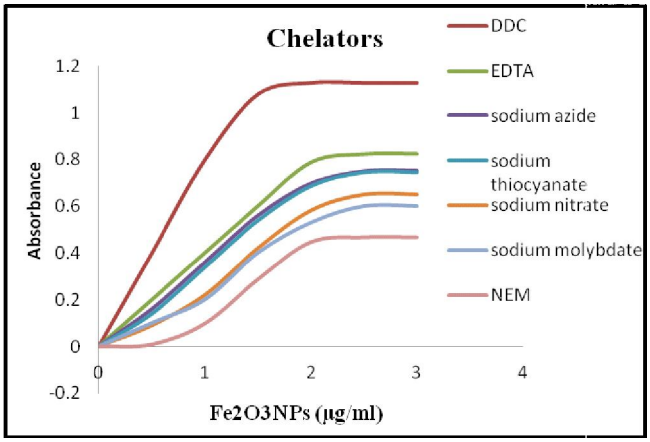

Fig. 5: Effect of chelators on activity of xanthine oxidase (XO) *in-vitro* in presence of green Fe_2O_3-NPs.

considered as 100% in both *in-vitro* and human serum. When green Fe_2O_3-NPs was incubated at different concentrations *in vitro*, the XO activity was inhibited by 13% at 1, 10 and 20µg/ ml, 30% at 30µg/ ml, 55% (significant) at 40µg/ ml, 60-80% at rest of the concentrations. Likewise chemical Fe_2O_3-NPs inhibited the XO activity by 50% (significant) at even low dose 1 to 10 µg/ ml, than inhibited in gradual manner from 55 % to 88 % at 30, 40, 50, 60, 70, 80, 90 and 100µg/ ml.

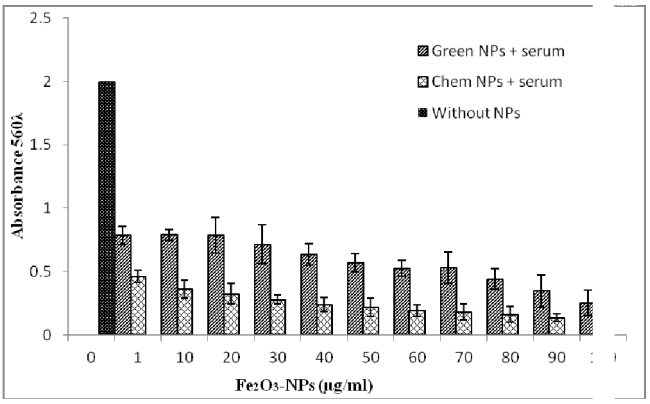

Fig. 2: Effect of Fe_2O_3-NPs on the activity of XO in human serum.

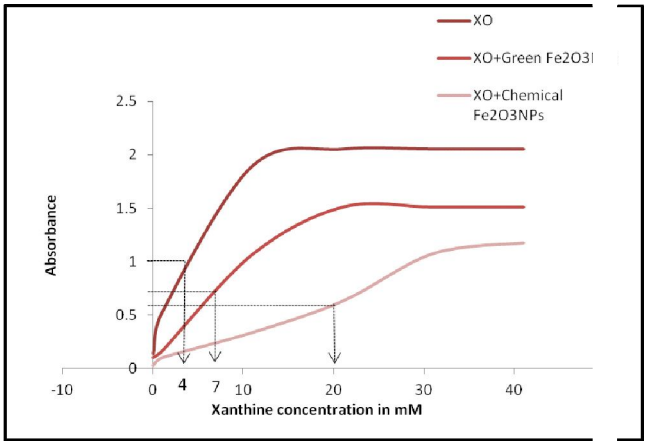

Fig. 4: Effect of substrate on activity of xanthine oxidase (XO) in-vitro, in presence of green and chemical Fe_2O_3-NPs.

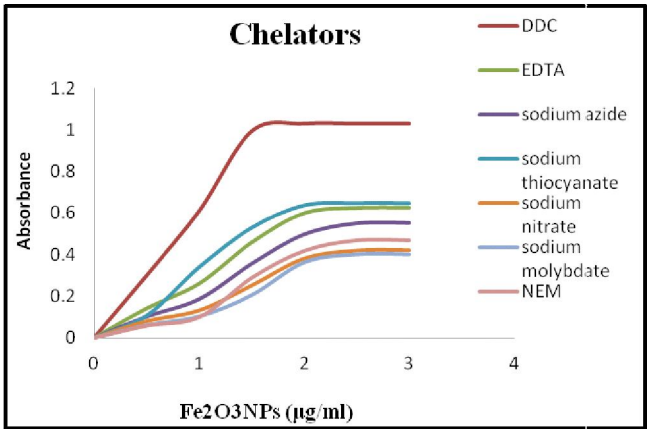

Fig. 6: Effect of chelators on activity of xanthine oxidase (XO) *in-vitro* in presence of chemical Fe_2O_3-NPs.

When green Fe_2O_3-NPs was incubated at different concentrations in used human serum, the XO activity was inhibited more than 55% (significant) even at very low dose 1 and 10 µg/ ml and also at 20 µg/ ml, than more than 60 % at rest of the concentrations upto 75% inhibition. Likewise chemical Fe_2O_3-NPs inhibited the XO activity 75% (significant) at even low dose 1µg/ ml, more

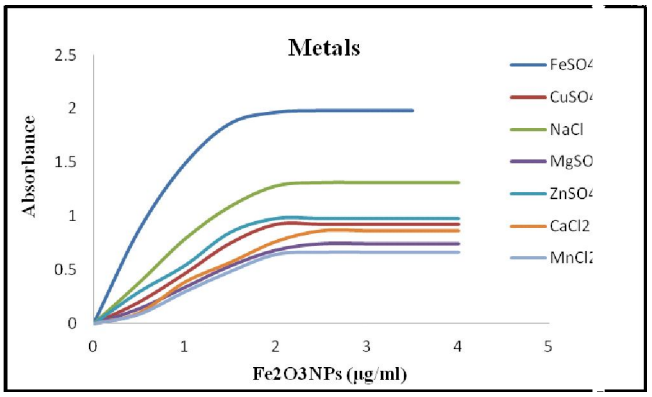

Fig. 7: Effect of metals on activity of xanthine oxidase (XO) in vitro in presence of green Fe_2O_3-NPs.

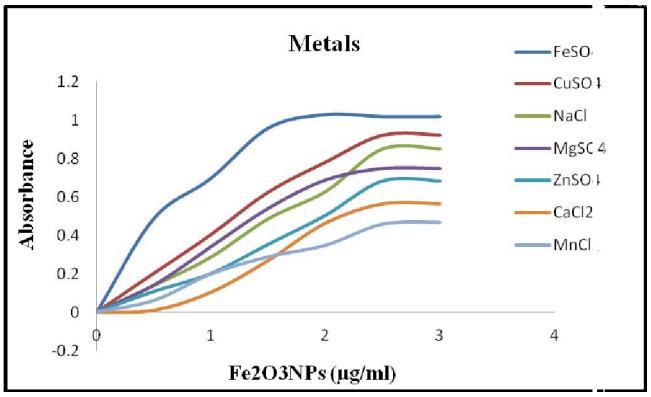

Fig. 8: Effect of metals on activity of xanthine oxidase (XO) in vitro in presence of green Fe_2O_3-NPs.

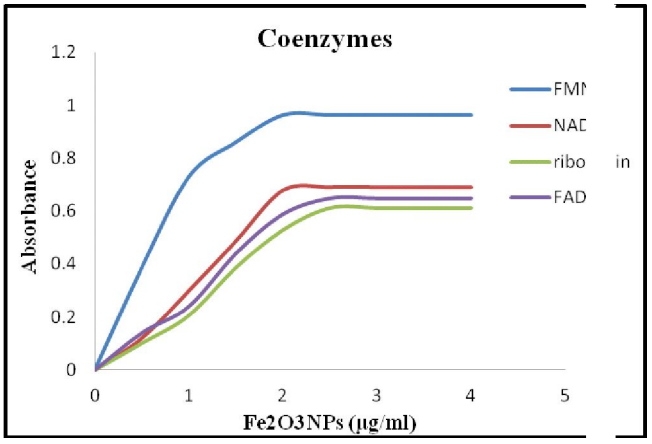

Fig. 9: Effect of coenzymes on activity of xanthine oxidase in presence of green Fe_2O_3-NPs.

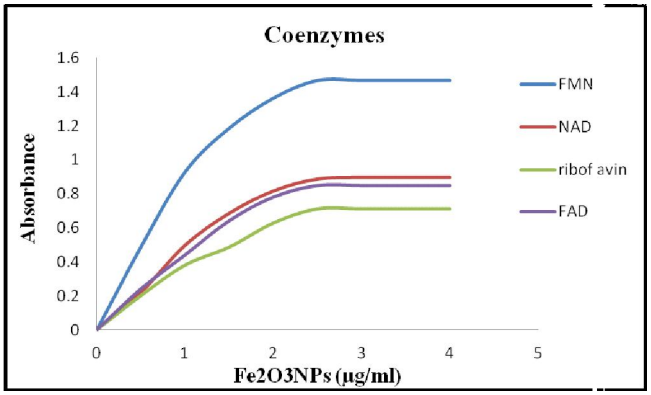

Fig. 10: Effect of coenzymes on activity of xanthine oxidase in presence of chemical Fe_2O_3-NPs.

than 75% at rest of the concentrations (Fig. 2).

The XO activity was decreased as the concentration of Fe_2O_3NPs was increased which might be due to the interaction of Fe_2O_3NPs with the surface of enzyme through H-bonding (Esfandfar *et al.*, 2016; Chauhan *et al.*, 2013). Such a binding of Fe_2O_3NPs with the enzyme could bring the change in the conformation of enzyme and hence the activity of enzyme is decreased as the concentration is increased.

Effects of Fe_2O_3NPs on kinetic properties of XO

Effect of pH

XO showed arise in optimum pH from 7.4 to 8.0 and 8.9, when incubated with green and chemical Fe_2O_3NPs respectively (Fig. 3). The increase in optimum pH of the enzyme in presence of Fe_2O_3NPs could be due to release of Fe^{2+} from Fe_2O_3NPs, which might deprotonate buffer (Chauhan *et al.*, 2013).

Effect of incubation temperature

XO showed no significant change in its optimum temperature (40 ^{0}C), when incubated with both commercial and green Fe_2O_3NPs.

Effect of substrate concentrations

The effect of varying xanthine concentrations (0.01–50) on free XO was shown in Fig. 4. The initial velocity of free XO was a hyperbolic curve, in the concentration range of 0.01 to 10 mM. The effect of xanthine in presence of green Fe_2O_3NPs was hyperbolic in the concentration range 1 to 20 mM but this effect was parabolic in presence of chemical Fe_2O_3NPs in the higher concentration range 20 to 32 mM, after which the reaction rate was constant as shown in Fig. 4. Km value for XO in the presence of green Fe_2O_3NPs was 7 mM. However Km value in the presence of chemical Fe_2O_3NPs was 20 mM, which was much higher than Km of native enzyme (4 mM). The increase in Km shows the decreased affinity of enzyme in the presence of Fe_2O_3NPs, which might be due inhibition of enzyme most probably non-competitive.

Effect of metal chelators

The addition of sodium azide, NEM, EDTA, sodium molybdate, sodium nitrate, sodium thiocyanate and DDC individually, in the reaction mixture, were unable to safe guard the enzyme from inhibition done by green/chemical Fe_2O_3NPs (Fig. 5 & 6).

Effect of metals

Among the various metal salts tested such as NaCl, $MnCl_2$, $CaCl_2$, $ZnSO_4$, $MgSO_4$, $FeSO_4$, and $CuSO_4$ at 1.0 mM concentration, only $FeSO_4$ caused slight stimulation of enzyme. It might occur due to the possibility that Fe SO_4 salt may react to the Fe-S cluster of XO enzyme and thus stimulate the electron transport (Fig. 7 & 8) in presence of green/chemical Fe_2O_3NPs.

Effect of coenzymes

Of the coenzyme tested, only FAD, at 1.0 mM concentration in the presence of Fe_2O_3NPs stimulated the activity, similar to native enzyme. Comparatively green Fe_2O_3NPs stimulated the enzyme activity significantly more than the commercial Fe_2O_3NPs. There may be the possibility that the plant biomolecules such as proteins, carbohydrates and secondary metabolites like flavonoids, terpenoids and phenolic compounds that are attached to NPs interact better than the chemical NPs (Fig. 9 & 10).

Conclusion

Fe_2O_3NPs which was green synthesized inhibit the XO activity comparatively lower than that by chemical Fe_2O_3NPs, when added in reaction mixture and serum in a spectrophotometric assay at rate of 1, 10 and 20ug/ml. But the high dose of both green and chemical Fe_2O_3NPs (90 and 100 ug/ml) caused nearly complete inhibition of XO activity. Km value of XO was increased upon addition of both Fe_2O_3NPs indicating the lower affinity of enzyme towards the substrate.

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