Chapter 6
Iron Oxide Nanozyme in Biomedicine

Amit Kumar, Diptikanta Acharya, Sagarika Satapathy, and Sushil Kumar Sahu

Abstract Iron oxide nanoparticles with intrinsic enzymatic properties are considered as iron oxide nanozymes (IONzymes). IONzymes are bridging the gap between nanotechnology and biomedicine. Their catalytic actions mimic those of natural enzymes such as peroxidase and catalase. IONzymes are synthesized chemically and are less expensive than the preparation of natural enzymes. They are stable over a wide range of pH and temperature, an advantage over natural enzymes. In addition to IONzyme's enzymatic properties, their magnetic properties provide opportunities for developing of bioseparation assays, imaging tools, targeted drug delivery and hyperthermia therapy in the field of biomedicine.

Keywords IONzymes · Nanotechnology · Biomedicine · Peroxidase · Catalase · Drug delivery

6.1 Introduction

Iron oxide particles show the properties of remanence and coercivity. Remanence refers to the amount of magnetization retained by iron oxide at zero driving field, and coercivity is the amount of driving field needed to demagnetize it [1]. Iron oxide nanoparticles are paramagnetic or superparamagnetic in nature, which is a superior characteristic over iron oxide particles [2]. So, they quickly aggregate and re-disperse by applying and removing an external magnetic field, respectively [3]. Due to this property, iron oxide nanoparticles have multiple biomedical applications,

A. Kumar
Department of Human and Molecular Genetics, Virginia Commonwealth University, School of Medicine, Richmond, VA 23298, USA

D. Acharya · S. Satapathy
Department of Biotechnology, GIET University, Gunupur, Rayagada, Odisha 765022, India

S. K. Sahu (✉)
Department of Zoology, Siksha-Bhavana, Visva-Bharati University, Santiniketan, West Bengal 731235, India
e-mail: sushilkumar.sahu@visva-bharati.ac.in

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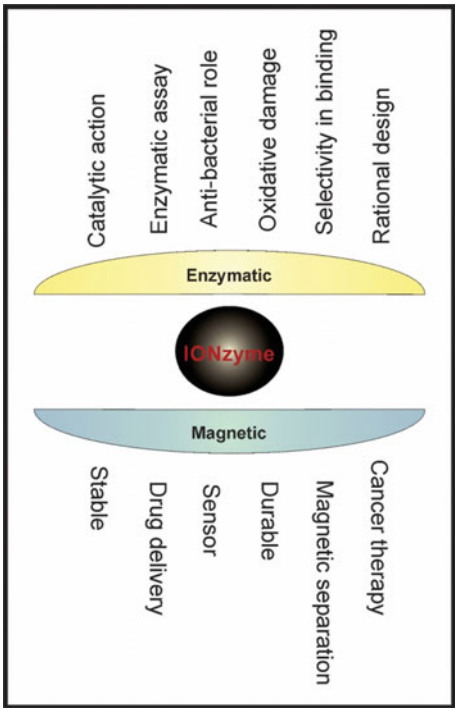

Fig. 6.1 Enzyme-like activities and magnetic properties of iron oxide nanoparticles (IONzymes) for biomedical applications

which include but are not limited to biosensors [4, 5], bioseparation [6, 7], targeted drug delivery [8, 9], magnetic resonance imaging [10] and hyperthermia therapy [11, 12]. Haematite (Fe_2O_3) and magnetite (Fe_3O_4) nanoparticles are considered as iron oxide nanozymes (IONzymes) due to their enzymatic properties. The first reported nanozyme was Fe_3O_4 nanoparticles, which possess horseradish peroxidase (HRP)-like catalytic activity [13]. Subsequently, different metals and metal oxides having enzymatic activities similar to peroxidase, oxidase, catalase and superoxide dismutase were studied [14–16]. Here, we present the catalytic activities of IONzyme and their applications in biomedicine (Fig. 6.1).

6.2 Enzymatic Activities of IONzyme

IONzyme is considered an enzyme mimetic as it possesses catalytic mechanisms similar to natural enzymes [17]. They mimic the properties of enzymes such as peroxidase and catalase of the oxidoreductase family [13, 18]. Both contain a non-protein part (or cofactor), i.e. haem. Peroxidase acts on hydrogen peroxide (H_2O_2) and gives rise to free radicals, whereas catalase acts on H_2O_2 to give rise to oxygen. Both of them play a crucial role in preventing oxidative damage in aerobic organisms.

(1) **IONzymes as peroxidase:**

IONzymes (nanomaterials of Fe_2O_3 and Fe_3O_4) mimic peroxidase activity (Eq. 6.1). Fe_3O_4 nanomaterial has been reported to exhibit better activity than Fe_2O_3 nanomaterial [19, 20]. IONzymes show optimum peroxidase activity at 37–40 °C in an acidic (pH 3–6.5) medium [18]. They can act on polysaccharides, lipids, proteins, and nucleic acids and peroxidize all these substrates [19, 21]. Activators of IONzymes are AMP, ADP and ATP [22], and inhibitors are free radical quenchers such as sodium azide, ascorbic acid, hypotaurine and catecholamines [17, 23].

$$H_2O_2 \xrightarrow{\text{Peroxide}} 2A + 2H_2O \tag{6.1}$$

(2) **IONzymes as Catalase:**

IONzymes also mimic catalase activity (Eq. 6.2). It has been shown that maghemite (γ-Fe_2O_3) and Fe_3O_4 nanoparticles decompose H_2O_2 both at neutral and basic pH [18, 19].

$$2H_2O_2 \xrightarrow{\text{Catalase}} O_2 + 2H_2O \tag{6.2}$$

6.3 Kinetics and Mechanism of Action of IONzyme

IONzymes follow Michaelis–Menten kinetics as in Eq. 6.3 [24].

$$v = (V_{max}[S])/(K_m + [S]) \tag{6.3}$$

where 'v' is the initial velocity of the reaction, 'V_{max}' is the maximal rate of the reaction, '[S]' is the concentration of the substrate, and 'K_m' is the Michaelis–Menten constant.

$$K_{cat} = V_{max}/[E] \tag{6.4}$$

where 'K_{cat}' is the catalyst rate constant that describes the limiting rate of any enzyme-catalysed reaction at saturation (Eq. 6.4).

The determinations of V_{max}, K_m and K_{cat} for IONzymes are apparently determined based on the peroxidase reaction. Tetramethyl benzidine-hydrogen peroxide (TMB-H_2O_2) is a chromogenic substrate for HRP. This substrate produces a soluble blue colour in the presence of HRP. This reaction can be stopped with an equal volume of 1N sulfuric acid. The optical density of the resulting yellow colour can be read at 450 nm. HRP can be replaced with IONzyme, which has a greater affinity for TMB than the native enzyme. IONzyme's surface has abundant iron in contrast to the one iron in the HRP molecule, which may be attributed to its higher affinity.

For the measurement of catalase activity, oximetry is preferred. An oxygen electrode senses the rate of O_2 generation. The reaction rate of catalase is positively correlated with the amount of molecular oxygen generated in the solution. However, it may be affected by external factors such as temperature, diffusion and oxygen in the air. Catalase can be replaced with IONzyme to perform the above process [25, 26].

A haem group and a coordinated iron are present in the active sites of the enzymes peroxidase and catalase. This helps with electron transfer during redox reactions. In an IONzyme, the superficial surface may act as an active site. The affinity of IONzyme towards H_2O_2 can be enhanced by manipulating the surface of IONzyme by molecular coating, imprinting or grafting other substances on its surface [24, 27].

6.4 Synthesis of IONzyme

IONzyme synthesis is achieved using the chemical methods, co-precipitation, solvothermal preparation, sol–gel, oxidative hydrolysis, thermal decomposition and Massart hydrolysis [27]. However, shape, size, morphology, nanostructure and activity change from one method to another. Biogenic methods of IONzyme synthesis like bacterial magnetosomes are possible, and they produce uniform-size IONzymes with better dispersity and biocompatibility than chemical methods [28, 29]. Additionally, modification of IONzyme's surface or its integration into other substances can be done to form multifunctional hybrid nanocomplexes to facilitate its further applications. For example, iron oxide is integrated onto the surface of graphene oxide and hydrogel [30, 31]. It is hard to compare IONzymes produced by different available methods and find the best one.

6.5 Properties of IONzyme

(1) **Stability**: IONzymes are more stable in comparison to natural enzymes like peroxidase and catalase in a broad range of pH and temperature. For instance, Fe_3O_4 nanozyme is stable at pH 1–12, with temperatures 4–90 °C although the catalytic activity reduces at a pH below 5 and above 40 °C [32–34].

(2) **Tunability**: IONzymes can be tuned to enhance their activity by modulating their shape, size and surface. Usually, the smaller the size of IONzymes, the superior the catalytic activity [35]. The activity of IONzymes could be improved by doping with other elements like gold (Au), silver (Ag) and platinum (Pt). Au–Fe_3O_4 nanoparticles (NPs) have more peroxidase-like activity than Fe_3O_4 NPs due to the synergistic effect of Fe_3O_4 NPs and Au NPs. Also, polarization effects from Au to Fe_3O_4 occur. Ag-nanowire coated on Fe_3O_4 NPs gives enhanced peroxidase-like activity compared to Fe_3O_4 NPs. Pt–Fe_3O_4 NPs show enhanced catalytic activity compared to Fe_3O_4 nanoparticles. Iron oxide NPs integrated

into carbon nanomaterials exhibit better peroxidase activity than pure iron oxide nanoparticles [24, 36, 37]. Surface modifications of IONzyme may substantially increase, decrease or reduce its activity depending upon the microenvironment and nature of the substrate. Fe_3O_4 NPs modified with polyethylene glycol decrease enzyme activity, but modifications with dextran have no substantial effect. Likewise, the surface charge of IONzyme may increase or decrease its activity. Heparin-coated negative surface charges of iron oxide nanoparticles showed nearly sixfold higher peroxidase activity than those with ethyleneimine-coated positive surface charges using TMB as the substrate. However, positive surface charges showed more than 11-fold higher peroxidase activity than those with negative surfaces using 2,2′-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) as the substrate. ABTS is a chromogenic substrate for HRP that yields a green end product after reacting with peroxidase. Catalytic efficiency was further increased by lowering the size of NPs [24]. Integration of biomolecules can enhance the IONzyme's activity. For instance, the peroxidase-like activity of DNA-capped iron oxide nanoparticles and casein-coated magnetic nanoparticles has more effect than that of naked nanoparticles using the TMB system [38].

(3) **Multifunctionality**: IONzyme has the property of superparamagnetism, and enzymatic activity is an additional nanoscale feature. Hence, both of these properties allow IONzymes for multi-purpose performance. IONzyme mimics peroxidase activity in acidic pH and catalase activity in neutral pH [19]. So, these activities can be regulated by changing pH in a cancer microenvironment or a biofilm formed by dysbiotic microbiota. Moreover, IONzyme can be used as a vehicle to load numerous molecules into a pathway to perform cascade reactions [39].

6.6 Applications of IONzyme in biomedicine

In the area of biomedicine, the enzymatic activity of IONzyme has been widely reported. The peroxidase-like activity of IONzyme provides an opportunity for designing colorimetric assays. Free radicals generated by it help kill microbes and cells via the ROS-mediated signalling pathway.

(1) **Enzyme alternative for immunoassay and pathogen detection**

IONzymes can be used instead of peroxidase in assays based on peroxidase activity. For example, IONzymes can be use in place of HRP-based detection in enzyme-linked immunosorbent assays (ELISA) and other similar assays by conjugating antibodies to them (Fig. 6.2).

Similarly, the superparamagnetism properties of IONzyme help enrich a small amount of antigens and improve the sensitivity of the assay. For example, chitosan-modified magnetic nanoparticles successfully detect a very small amount (1 ng/ml) of carcinoembryonic antigen. This immunoassay has been reported to detect

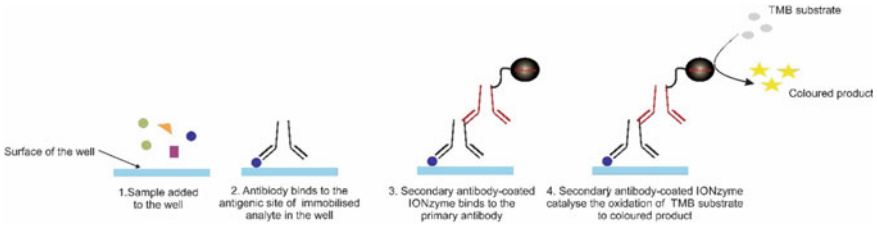

Fig. 6.2 IONzymes for immunoassay. The antibody recognizes the antigenic site of the analyte and is then detected by the second antibody-coated IONzymes, which catalyse the oxidation of colorimetric (TMB) substrates to give rise to a coloured product

IgG, human chorionic gonadotropin, epidermal growth factor receptor and human epidermal growth factor receptor [18]. IONzyme can be used to develop a lateral flow-based diagnostic test kit. This has been successfully achieved to detect glycoproteins of the Ebola virus and is more sensitive than the standard colloidal gold strip. A similar method has been used to detect the new bunyavirus. IONzyme's surface is coated with a forward primer and a biotinylated reverse primer, which can amplify the target DNA during PCR. This can bind to a streptavidin-coated surface and produce a signal via the catalysis of IONzyme. This assay can detect *Vibrio cholerae* with a minimum of 10^3 colony-forming units/mL and show directions for detecting other bacterial DNAs in food and water. IONzyme's surface, coated with aptamers, has the potential to identify target molecules with high specificity. This method has been reported to detect *Listeria monocytogenes* in food [40].

(2) **Enzyme cascades and substrate-based detection**

Multiple enzymes can be brought together onto the IONzyme surface to execute a cascade of enzymatic reactions to react with the substrate, followed by its detection indirectly by analysing the product. Glucose oxidase was bonded with Fe_3O_4 nanoparticles. Then, glucose was catalysed by glucose oxidase to produce H_2O_2. H_2O_2 was catalysed by IONzymes due to its peroxidase activity. By using a chromogenic substrate, a colour signal can be formed in proportion to the concentration of glucose [19, 39]. This glucose detection method was reflected in many other reports [24, 41, 42]. Similarly, different oxidases that can produce H_2O_2 as an intermediate in a cascade of reactions can be bonded to IONzyme to detect the corresponding substrates. In this regard, galactose oxidase for galactose, cholesterol oxidase for cholesterol and alcohol oxidase for alcohol have been reported to be used in IONzyme base detection [43, 44]. Integrating of a series of natural enzymes into IONzyme to participate in a cascade reaction offers a novel approach to developing assays for detecting any molecules present in the cascade.

(3) **Diagnosis of tumour and its therapy**

IONzyme shows promising applications for the diagnosis of tumours and their therapy. For tumour diagnosis, an IONzyme called magnetoferritin (MFt) nanoparticles, a superparamagnetic protein, can be used. MFt nanoparticles were encapsulated inside the shell of human heavy-chain ferritin. Upon delivery, this binds to

cancerous cells overexpressing transferrin receptor 1. The peroxidase activities of IONzyme allow the oxidation of target substrates in the presence of H_2O_2, resulting in the formation of coloured products. The coloured product can be seen in the solid tumour [45]. In a therapeutic approach, IONzyme can be used to kill cancer cells by catalysing H_2O_2 and producing toxic radicals in the tumour microenvironment. For this, delivery of H_2O_2 into the target tissue in vivo or integrating an enzyme that can produce H_2O_2 using a substance in the target tissue as a substrate may be considered. This approach has been tested using Fe_3O_4 nanozyme and H_2O_2 in a mouse model of cervical cancer [46]. Iron present in the nanomaterials produces a lot of ROS, resulting in the death of cancer cells, or induces polarization of macrophages in tumour tissues to reduce their further growth without administration of H_2O_2 from outside [47, 48]. Such anti-cancer effects may be attributed to the enzymatic action of iron in nanomaterials, which is similar to IONzyme. It is crucial to understand the safety of IONzyme in terms of its distribution, kinetics, action and clearance in an animal model. Fe_3O_4 nanoparticles coated with dextran were localized mostly in the liver, lung and spleen and less in the kidney, lymph nodes and thymus. Usually, nanoparticles are taken up by the reticuloendothelial system and circulated to the liver, lung and spleen.

(4) **Anti-bacteria and biofilm elimination**

IONzymes have the potential to kill bacteria and reduce biofilm formation [49]. H_2O_2 gives rise to free radicals in the presence of IONzyme, which can destroy bacterial cells by attacking their membrane proteins or genetic material in the nucleus. The free radicals inhibit bacterial biofilm formation. The peroxidase-like activity of IONzyme facilitates increasing the anti-bacterial action of H_2O_2. The increased effect has been reported on *Escherichia coli* and *Staphylococcus aureus* [46, 50]. These anti-bacterial properties are helpful to kill multiple-drug-resistant bacteria, inhibit sepsis and heal injuries. Free radical production and peroxidase activity in the presence of Fe_3O_4 nanozyme lead to oxidative damage of the components of biofilm, such as oligosaccharides, nucleic acids and proteins. The action of IONzyme and H_2O_2 independently is not efficient, but synergistic effects work better for oxidative degradation [18]. The Fe_3O_4 nanoparticles and H_2O_2 work as a system to cleave biomolecules, resulting in the degradation of existing biofilms and preventing the formation of new ones. This approach has been reported to target microorganisms in the oral microenvironment to inhibit the formation of plaque and dental caries [51]. The peroxidase-like action of IONzyme breaks glucans in the biofilm matrix into glucose and kills microbes like *Streptococcus mutans*. It also reduces the demineralization of teeth in an acidic environment created by dysbiotic microbiota. These reports suggested the potential of nanozymes as a compelling option for managing of biofilm-related illnesses.

6.7 Conclusion

IONzyme is regarded as an enzyme mimetic of the new generation due to its strong catalytic properties. The kinetics of IONzyme were subsequently investigated to elucidate its mechanism of action and improved by manipulating its size, shape, surface, dopants and combination with other nanoparticles. This facilitates sensibly designing the appropriate nanozymes for practical appliances. Compared to natural enzymes and other mimetic of natural enzymes, IONzymes are more stable. Also, they are multifunctional and versatile since they can be modified by additional labelling to function on multiple platforms in terms of assay development and therapy. These properties have shown a new direction for using magnetism-independent iron oxide nanomaterials. It has been reported that magnetic iron oxide materials can be used in the field of biomedicine in some specific instances, like DNA isolation, delivery of genes to the target, sorting of desired cells and imaging of solid tumours. For instance, we can sort T-cells to be used in chimeric antigen receptor (CAR) T-cell therapy, an important immunotherapy for treating cancer. These uses are based on their magnetic properties. IONzymes, to which the enzyme-like properties bring further advantages like immunoassays, detection of microbes, diagnosis and therapy of tumours, biofilm removal, and free radical modulations at different levels for cellular differentiation and development. As yet, several IONzymes and their applications have been addressed, but several insight challenges still need to be addressed. A standard procedure is needed to calculate and compare the activities of different IONzymes from independent preparations. Specific activity is one way to evaluate enzyme activity. More specific evaluation is needed for calculating the K_M, K_{cat} or K_{cat}/K_M in the same reaction conditions in terms of substrate quantity, temperature, pH and buffer.

IONzymes are not natural, and their interaction, affinity and action are not the same as those of natural enzymes; the method to improve their selectivity is still incomplete. The feature of interaction at the molecular level between enzyme and substrate may help to synthesize IONzymes with enhanced selectivity. Molecular imprinting is one way to improve the IONzyme's selectivity. Enzyme-mimicking activities of IONzyme are studied mostly in catalase and peroxidase. Numerous natural enzymes utilize iron as a cofactor to execute catalytic activities. Therefore, it is vital, though difficult, to design IONzyme with a preferred action. The probable clue remains in understanding the detailed structure and functions of natural enzymes. In vivo activities of IONzyme and their correlation with catalytic activity, therapeutic effect, and biocompatibility still need to be clearly understood. Iron oxide nanoparticles got clinical permission for magnetic resonance imaging (MRI) of tumours in vivo. However, its biocompatibility and intrinsic peroxidase and catalase-like activities need to be carefully evaluated. Influences on reactive oxygen species-sensitive events in vivo need to be investigated. These include immune activation and repression, the development of nerves and the nervous system, heart regulation, stem cell lineage maintenance, differentiation and growth. Overall, great efforts are needed to address and overcome the fundamental challenges and advance IONzyme's activity for both in vitro and in vivo applications.

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