CHAPTER 2

Literature reviews

2.1 Organophosphate pesticides

Pesticides constitute a heterogeneous category of chemicals specifically designed for the control of pests, weeds or plant diseases. OPs are esters of phosphoric acid and its derivates. The general chemical structure of an organophosphate (Figure 2.1-2.2) comprises a central phosphorus atom (P) and the characteristic phosphoric (P=O) or thiophosphoric (P=S) bond. The symbol X represents the leaving group, which is replaced (by nucleophilic substitution) by the oxygen of serine in the AChE active site. In very toxic warfare agents the leaving group contains fluorine (F), which has high tendency to hydrolysis and thus extremely high AChE inhibition. In less toxic OPs the leaving group usually contains alkyl or aryl groups [6]. Commonly used OP includes parathion, malathion, methyl parathion, chlorpyrifos, diazinon, dichlorvos, phosmet, fenitrothion, tetrachlorvinphos and azinphos methyl (Figure 2.3) [7-8].

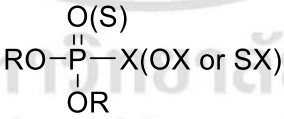

Figure 2.1 General structures of OPs [8]

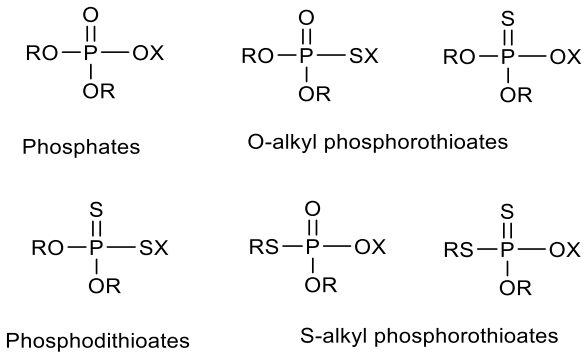

Figure 2.2 Main types of OPs (R is usually methyl or ethyl group and the leaving group X is aliphatic, homocyclic or heterocyclic) [8]

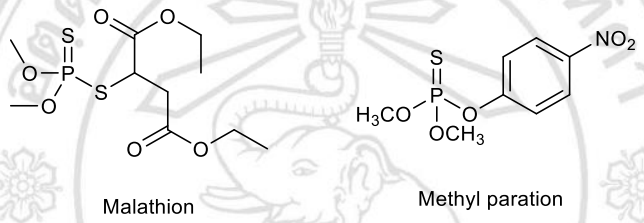

Figure 2.3 Chemical structure of some OPs compounds [9]

2.2 Toxicity of OPs

The primary mechanism of OPs toxicity is the irreversible inhibitory effects on cholinesterase which is an enzyme essential for the proper functioning of the nervous system of vertebrates and insects. The inactivation of the AChE occurs in the blood and in a wide range of nerve, neuromuscular (skeletal, smooth and cardiac) and glandular tissues where these enzymes have a role in cell-to-cell communication and the hydrolysis of xenobiotics [10]. The toxic action of OPs arise from the inhibition of acetylcholinesterase activity as shown in Figure 2.4, leading to accumulation of acetylcholine at the nerve endings and therefore causing cholinergic overstimulation characterized by severe consequences in humans including abdominal cramps, muscular tremor, hypotension, breathing difficulty, diarrhea, slowing heartbeat muscular fasciculation, and paralysis and even death [11-12].

Acetylcholine is a neurotransmitter active in central nervous systems and skeletal muscle junction. AChE belongs to the family of carboxylesterase and it is concentrated at neuromuscular junctions and cholinergic brain synapses. It is hydrolase that degrades

acetylcholine molecules into choline and acetic acid, thus terminating impulse transmission at cholinergic synapsis. Therefore, AChE controls generation of nerve impulses in the postsynaptic neurons. Toxicity of OPs depends on inhibition of AChE, thus the enzyme is a common bioevaluator for the detection of OPs. Extension of inhibition of this enzyme has been frequently used to measure quantitatively the presence OPs [13-14]. The onset, severity and duration of poisoning is determined by the dose, route of exposure, physicochemical properties of the OPs (e.g. lipid solubility), rate of metabolism (whether transformation in the liver is required before the compound becomes toxic) and whether the organophosphorylated cholinesterase ages rapidly as it is shown in Figure 2.5 [15].

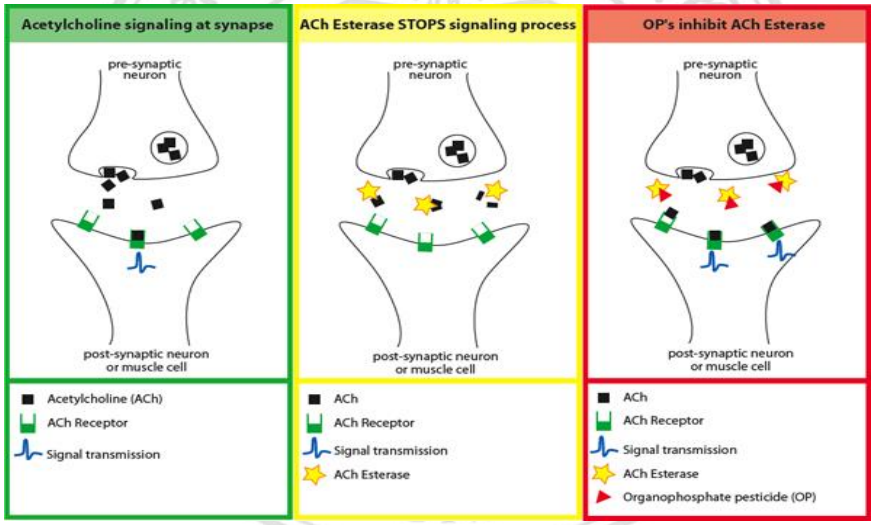

Figure 2.4 Mechanism of OP toxicity [15]

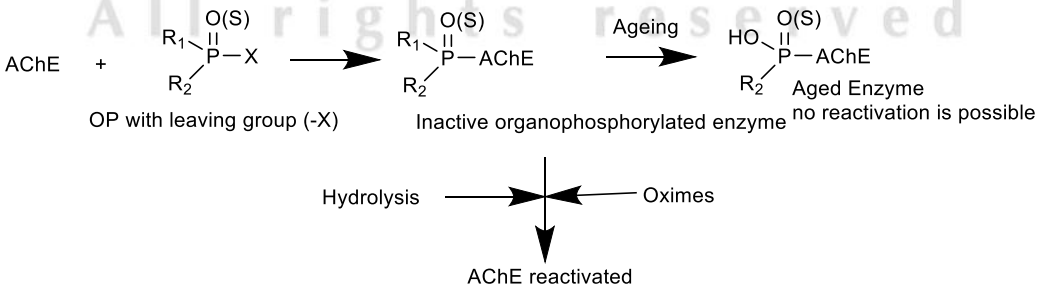

Figure 2.5 Key reactions occurring between OP and AChE [15]

2.3 Effect of OPs [10]

The following effects of OP agents have been demonstrated in animals and are theoretically possible effects in man including;

2.3.1 Inactivation by phosphorylation of other beta esterases.

2.3.2 Altering the release of neurotransmitters, e.g. γ-aminobutyric acid (GABA) and glutamate.

2.3.3 Increasing the number of GABA and dopamine receptors.

2.3.4 Acting as agonists at M2/M4 muscarinic receptors.

2.3.5 Inhibition of mitochondrial enzymes, respiration and ATP generation.

2.3.6 Induction of mast cell degranulation, probably causing the release of histamine or histamine-like compounds.

2.3.7 Inhibition of nitric oxide.

2.3.8 Interference with surfactant in the lung.

2.3.9 Inhibition of phospholipase A_2.

2.3.10 Interference with humoral and cellular immunity, e.g. the function of T lymphocytes.

2.4 OPs

In this research, two OPs, namely dimethoate and profenofos were used for detection of their acetylcholinesterase(AChE) inhibitory activities.

2.4.1 Dimethoate

Dimethoate is a broad use, systemic OP insecticide and acaricide which has been in use since 1956 against insects and mite on agricultural crops and ornamental plants. Dimethoate is primarily recognized for its neurotoxic effects caused by inhibition of AChE by its oxon metabolite, dimethoxon [16]. The structure of dimethoate as it is shown in Figure 2.6.

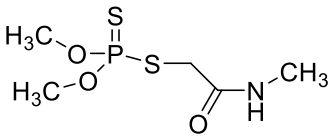

Figure 2.6 Structure of dimethoate [16]

2.4.2 Profenofos

Profenofos is a thiophosphate OPs (O=P-S-C) that was developed for pest strains resistant to chlorpyrifos and other OPs. It is selectively toxic to insects compared to mammals due to different metabolism of the propylthiol group. It has also been classified as a moderately hazardous (Toxicity Class II) pesticide by the World Health Organization (WHO) and it has a moderate level of acute toxicity following oral and dermal administration [16-17]. The structure of the profenofos is as it is shown in the Figure 2.7.

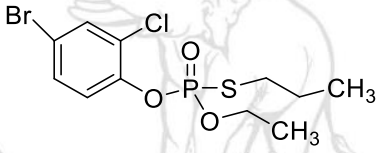

Figure 2.7 Structure of profenofos [17]

2.5 Detection of OPs

Detection and quantification of the pesticides has become vital important in the recent years due to their exhaustive use in the agriculture. Number of analytical methods are in place for OP determination including, gas chromatography (GC), ion chromatography (IC), high performance liquid chromatography (HPLC), Fourier transform infrared spectroscopy (FTIR), enzyme-linked immunosorbent assay tests and colorimetric methods. Although chromatographic techniques are very accurate, they are time consuming and require extensive sample preparation [18-25]. Among those technologies, colorimetric method have become of huge interests due to their low cost instruments, simplicity, portability and practicality. In addition, colorimetric assays can be easily performed without the requirement of expensive or sophisticated instruments

and can be applied to the field analysis. However, the key challenge for colorimetric assay is the alteration of the detection procedures into color changes [24].

2.6 Determination of OPs using mass spectrometric techniques.

Mass spectrometry (MS) is the analytical technique in which the mixture of gaseous. Ions are separated according to their mass-charge *m/z* ratios. All mass spectrometers have an ion source, a mass analyzer and an ion detector. The nature of these components varies based on the type of mass spectrometer, the type of data required, and the physical properties of the sample. Samples are loaded into the mass spectrometer in liquid, gas or dried form and then vaporized and ionized by the ion source, both positive and negative ions can be studied [25-26], Diagram of mass spectrometer is as shown in Figure 2.8 below.

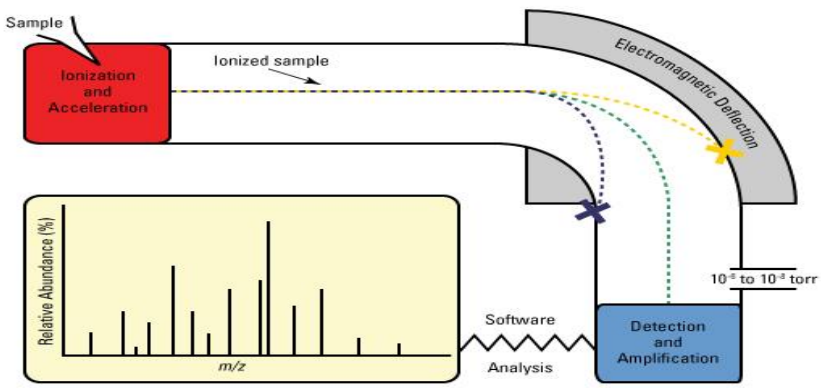

Figure 2.8 Diagram of a sector mass spectrometer [25]

MS techniques has been widely utilized for the determination of pesticides residues and their metabolites in foods to ensure safety of the food supply, investigation of the contamination of water resources from pesticides and their relevant metabolites and the structure elucidation of unknown metabolites or degradation products that sometimes can be more toxic than the parent pesticides due to their low detection limits in the part per billion (ppb) and part per trillion (ppt) range, as well as the selective detection of analytes in multi-residue samples. This techniqueis often coupled with separation methods like gas chromatography (GC) or liquid chromatography (LC), and other analyte introduction methods such as inductively coupled plasma (ICP), or fiber introduction (FI) [27].

2.6.1 Gas chromatography- mass spectrometry (GC-MS)

Gas Chromatography (GC) is a method for separating components of mixtures of volatile compounds. In most applications, the separations are made to identify and determine the quantity of each component of the sample of the mixture, and analytical gas chromatographic apparatus includes additional devices for this purpose. The instrument of the gas chromatography is consisting of carrier gas, flow control, sample injector, column, detector, recorder, collector and flow meter [25].

Gas chromatography- mass spectrometry (GC-MS) is a combination of two powerful analytical tools (Figure 2.9), Gas Chromatography for the highly efficient gas-phase separation of components in complex mixtures, and mass spectrometry for the identification of the unknowns [26]. GC-MS methods continue to play a crucial role in the field of pesticides residue analysis. A key advantage to the GC-MS is the availability of the revere search methods based on the commercially available electron impact ionization (EI) pesticide libraries along with automated mass spectral deconvolution software (AMDIS) allowing for rapid screening of the samples [25].

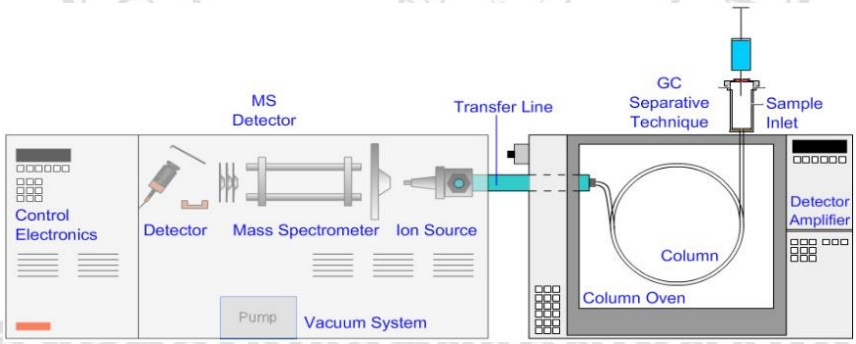

Figure 2.9 Schematic of a typical bench top GC-MS system [25]

Pinheiroet al., 2011, developed a rapid and simultaneous method for identification and quantification of pesticides residues in water samples and applied to the analysis of real samples. They applied a new single-drop micro extraction (SDME) followed by gas chromatography–mass spectrometry techniques were used to determine the dimethoate, methyl parathion, ethion and permethrin pesticides in water samples. They studied linearity, linear range, precision, accuracy, sensitivity and robustness as the parameters for validation of their SDME/GC–MS method. Recovery tests of pesticides in different water samples (tap water and river water) were between 76.2 and 107%. For all pesticides the method showed the limits of detection (LOD) in a range

between 0.05 and 0.38 μg L^{-1} and the limit of quantification (LOQ) between 0.15 and 1.1 μg L^{-1}. All these parameters validated high sensitivity of the developed method and the capability for detecting and quantifying of low levels of pesticides in water samples [1].

2.7 Biosensors for OPs screening

Biosensor comprises basically of three elements, that is biological recognition element, transducer, and signal detector as shown in Figure 2.10. The principle of biosensor systems, it comprises of the sensitive biological element (such as tissues, microorganisms, cell receptors, enzymes, antibodies, nucleic acids, etc.); the transducer or the detector element (physiochemical, optical, piezoelectric, etc.) that transforms the signal, resulting from the interaction between the analyte and the biological element into another signal that can be measured and quantified; and associated electronics or signal processors that are primarily responsible for the display of the results in a user-friendly way [27].

Biosensors are generally classified on the basis of the type of biological recognition elements (i.e., enzyme, antibody, nucleic acid, whole cell, etc.) and on the signal transduction method (optical, electrochemical, piezoelectric, etc.) used for detection. The biological recognition element must be extremely specific to the analyte for the accurate detection of the analyte in different samples. The sensitivity of biosensor relies on the biorecognition layer which catalyses the reaction [28].

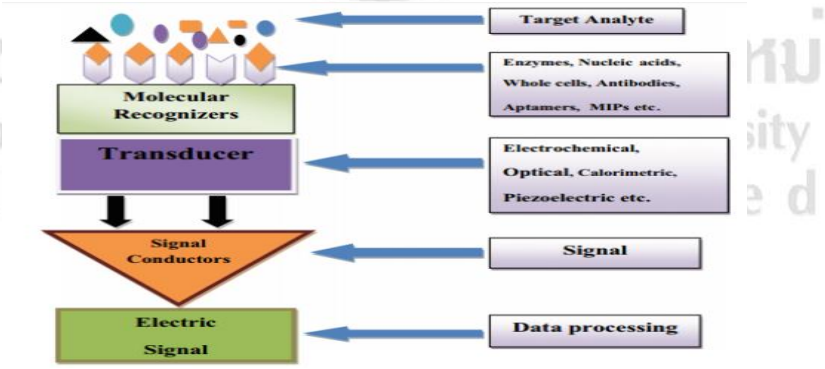

Figure 2.10 The principle of biosensor systems [28]

Biosensors emerge as a highly efficient analytical tool for the determination of above mentioned snarls linked with conventional techniques of pesticide detection. Biosensors pave way for easier and efficient pesticide detection with immense accuracy. The ability to detect very minute amounts of the target substance (pesticides), continuous monitoring, low cost, and decentralized infield analysis are the key features of biosensor technology. The close association of the biological events with the generation of a signal creates a path for developing compact and easy to use analytical tools of high sensitivity and specificity. Their biological base makes them ideal for toxicological measurement of pesticides (qualitative), while conventional techniques can only measure pesticides quantitatively [29].

Development of biosensor requires the immobilization of biosensing element with the interface of transducer. The immobilization secures both the stabilization of the biorecognition material and the proximity between the biorecognition material and the transducer. The immobilization method which are generally used are: physical adsorption at a solid plane, crosss-linkage between molecules, covalent attachment to a surface, entrapment within a membrane system or polymer as summarized in Table 2.1. Progress in the field of biosensors technology is mainly made by the upgrading of the biological components and the implementation of nanotechnology [29]

Table 2.1 Methods of immobilization of the biological component in biosensors

Method	Advantages	Limitations
Physical Adsorption	No modification of biological component is required	Binding is Susceptible to changes in pH and temperature
	Matrix can be regenerated	Binding is not strong
	Maximal retention of activity	Loss of biological activity
	Prepared simply and low cost	Treatment with toxic chemicals
Cross liking	Less chances of reduction of biological activity of biocomponent	High preparation cost
	Strong binding force	High running problem, Treatment with toxic chemicals.
Covalent binding	Strong binding force Less	High preparation cost
	No loss of biological activity	Difficult to prepare
	Less affected by adverse conditions	Matrix not regenerable
Entrapment	No direct chemical modification	Weak binding force
	Preparation cost moderate	Loss of biological activity
	Specificity retained	Applicable for small analyte detection

2.7.1 Enzyme based biosensors for OPs detection

Enzymes are one of the most appropriate choices as biosensing part in pesticide biosensors construction, because they possess high chemical specificity and have natural biocatalytic signal amplification property. In enzyme based biosensors, the biological element is the enzyme which is combined with a transducer. Enzymes react

selectively with its analyte and produce a signal proportional to the target analyte concentration. This signal can result from a change in proton concentration, release or uptake of gases, light emission, absorption or reflectance, heat emission, and so forth, brought about by the reaction catalyzed by the enzyme. The transducer converts this signal into a measurable response, such as current, potential, temperature change, or absorption of light through electrochemical, thermal, or optical means. This signal can be further amplified, processed, or stored for later analysis.

Enzymatic biosensors for the detection of pesticides are fabricated on the basis of two principles the one based on measurements of enzyme inhibition (indirect approach) or on direct measurement of compounds involved in the enzymatic reaction (direct approach). Most simple and innovative biosensor approach for the development of enzyme biosensors for pesticide detection is based upon the inhibition of enzyme AChE (cholinesterase). Two types of enzymes known AChE and butyrylcholinesterase (BChE) have been used [29].

AChE biosensors for OPs detection basically works on the inhibition effect in which the inhibition of acetylcholinesterase by various neurotoxins is monitored and the signal generated is inversely proportional to the concentration of OPs or, in other terms, an increased concentration of OPs leads to weak signals. These inhibition based sensors are very sensitive, but they offered poor specificity, as there are many substances such as carbamates, heavy metals, fluoride, nerve gas, or nicotine that also inhibit these enzyme. Advancement in fabrication strategies and methods of enzyme purification and its stabilization had astounded the drawbacks related to accuracy, sensitivity, and reliability making AChE based biosensors as a powerful tool for the rapid screening of OPs [8, 29]. Principle of working of acetylcholinesterase (AchE) based biosensor for Ops detection was shown in Figure 2.11.

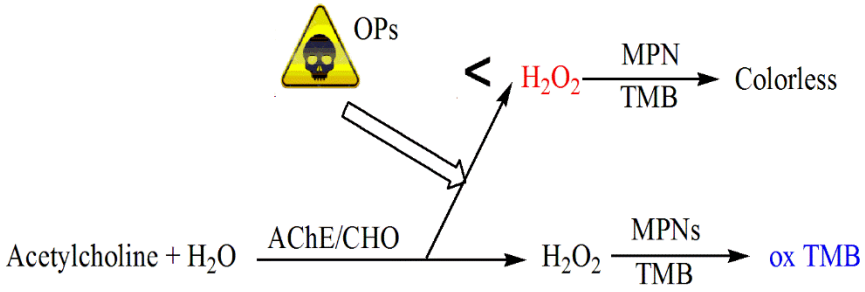

Figure 2.11 Principle of working of acetylcholinesterase (AchE) based biosensor for OPs detection [13]

2.8 Nanozymes used in OPs biosensing

The promising association between nanotechnology and sensors technology has come into play over the past few years. Origination of nanoscience technology and nanomaterials, has shown tremendous profitable aspects to the biosensors technology. Importance of these nanomaterials to the basic development of biosensors has been recognized. Nanomaterials have shown an immense capabilities to the further elaborative development to biosensors technology. Whenever materials scaled down to nanosize, surface area increases, and most of materials display some novel attributes that are not present in their large size counterparts [29].

Nanomaterials based biosensor developed from the association of material science, molecular engineering, and biotechnology, improve overall sensitivity and specificity of biomolecule recognition in various environmental samples. It also possesses the characteristic of manipulating atoms and molecules and has properties of recognition of biomolecules, pathogenic diagnosis, and environment monitoring. The immobilization of nanomaterialswith sensing part of biosensor generates novel interface thus enhances the sensitivity of the detection of analytes [29].

Nanozymes are nanoparticles with enzyme catalytic activity. Over the past few decades, researchers have established nanozymes as highly stable and low cost alternative to natural enzymes in a wide range of applications [29-31]. Since it was revealed that Fe_3O_4 magnetite nanoparticle (MPNs) possess intrinsic peroxidase activity and can catalyze redox type of reactions, in a way that similar to the peroxidases such as horseradish peroxidase (HRP), nanoparicles have received significant attention owing to their superior characteristics that include catalytic stability over a wide range of

temperatures and pH, controlled low cost large scale synthesis, and convenient separation by application of an external magnetic field and or by centifugation [32-33].

In 2007, Yan et al., Fe_3O_4 nanoparticles were prepared with three different particle sizes (30, 150 and 300 nm) and discovered that all these MPNs catalyzed the reaction of the substrate 3, 3, 5, 5-tetramethylbenzidine (TMB) to produce a blue color reaction in the presence of H_2O_2 with the maximumabsorbance of 652nm. Other peroxidase substrates such 3,3′-Diaminobenzidine (DAB) and *O*-Phenylenediamine hydrochloride (OPD) were also oxidized to their corresponding products, mimicking the activity of HRP. They also discovered that catalytic activity of these (nanozymes) was size dependent, with smaller sized particles displaying higher catalytic activity. Like HRP, they also discovered that nanozymes catalytic activity was dependent on pH, temperature and H_2O_2. Conversely compared with HRP, the Fe_3O_4 nanoparticles were much stronger as they remained stable and retain their catalytic activity after incubation at a wide ranges of temperature (4-90ºC) and pH (0-12) [34].

In 2012, Gu et al., also discovered that iron oxide nanozymes both Fe_2O_3 and Fe_3O_4 exhibited dual enzyme mimetic properties (i.e. catalase and peroxidase and peroxidase mimics) as it is shown in Figure 2.12. In their study, they discovered that iron nanozymes directly catalyzed H_2O_2 into water and oxygen in such a condition, named catalase-like activity in a neutral conditions (pH 7.4) and the peroxidase activities was dominant under acidic conditions at pH 4.8. while comparing their perfomance they also discovered that like for the peroxidase activities, Fe_3O_4 alone had a higher catalase activity than Fe_2O_3 [35].

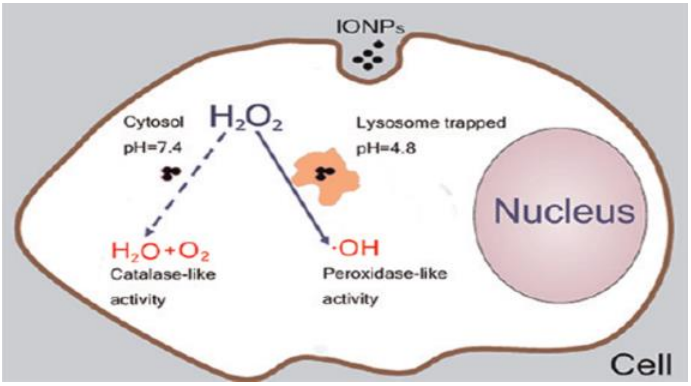

Figure 2.12 Dual enzyme like activities (catalase and peroxidase mimics) of iron oxide NPs [35]

Since then a considerable advance has been made in this area due to the tremendous progress in nanoresearch. Several other nanoscale materials, such as gold nanoparticles, rare earth nanoparticles and ferromagnetic nanoparticles, vanadium oxide (V_2O_5), cerium oxide, 16rapheme oxide and carbon nanotubes (CNTs) have been learnt to possess unique enzyme-mimic catalytic activities [36-37] as shown in Table 2.2

Table 2.2 Different types of nanoparticles used in colorimetric assays

S/N	Substrate/Nanoparticle	Analyte	Nanozymes
1	TMB - ɣ-Fe_2O_3	Human glucose	ɣ-Fe_2O_3
2	ABTS-ZnO-CNTs	Cholesterol	ZnO CNTs
3	TMB-O_2- Co_3O_4	Sulfite in foods	Co_3O_4
4	Fe_3O_4	Triacetate Triperoxide (TATP)	Fe_3O_4
5	ABTS - Fe_3O_4	Melamine in dairy Products	Fe_3O_4
6	AChE- Au	Organophosphorus	Au

2.9 Iron oxide nanozymes in OPs biosensing

Iron oxide nanomaterials and especially magnetic iron oxide nanomaterials have found broad use in many areas such as separation and capture of analytes, sensing and imaging. They are usually considered chemically and biologically inert so metal catalyst, enzymes, or antibodies are often conjugated for further functionalization [37].

In 2012, Liang, et al., developed Fe_3O_4 magnetic nanoparticle peroxidase mimetic-based colorimetric assay for the rapid detection of OPs and nerve agent using AChE and Choline Oxidase (CHO). In this work, it was found that AChE and CHO catalyzed the production of H_2O_2 in the presence acetylcholine which activated MPNs to catalyze the oxidation of calorimetric substrate TMB to produce a blue color reaction. The results showed that inhibition of AChE by OPs (acephate and methylparaoxon) and the nerve agent sarin prevented the production of H_2O_2, lead in reduction of catalytic oxidation of TMB and decrease in color intensity. Acephate, methylparaoxon and Sarin at the concentrations below 1 nM, 10 nM and 5 µM, respectively was detected which was environmentally more friends than the previous reported by AChE –based piezoelectric biosensor and Ellman's colorimetric assay [38].