

Review

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Review

Recent Developments on Colorimetric and Dual Colorimetric/Fluorimetric Enzymatic Biosensors for the Detection of Pesticides in Food

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Abstract: Pesticides are chemical products used to protect crops to prevent or eliminate undesirable organisms. Pesticides can contaminate continental waters, air, soil and food products. The presence of pesticide residues must therefore be monitored in water and food using sensitive, reliable and broad-spectrum analytical methods, as there is a huge variety of pesticides. Biosensors are one of the screening methods with great potential for detecting residues below the maximum residue limits. The different sensing principles of enzymatic biosensors for pesticide analysis will be described. This review highlights the great potential of colorimetric, enzymatic biosensors for detecting pesticide residues in water and food. Moreover the development of dual colorimetric and fluorimetric sensors during the past years is presented. These methods are most often based on the enzyme acetylcholinesterase, whose activity is inhibited by pesticides. The different approaches used in the development of these biosensors will be detailed, based on different chemical substrates, fluorophores and nanomaterials. The advantages and disadvantages of each approach will be discussed. Finally, the development prospects for improving these biosensors will be presented.

Keywords: colorimetric; dual ratiometric; biosensor; enzymatic; acetylcholinesterase; plant and animal derived-food

1. Introduction

Pesticides are used to protect plants, to control and destroy pests for plants. They could be classified from their spectrum of activity (ie. their target) (eg. Insecticides, acaricides, antiseptics, herbicides, etc), from their chemical hazards (from slightly to extremely hazardous) [1] or from their chemical structure and composition. Regarding the latest classification, pesticides belong to three main groups: inorganics compounds (eg. Chlorates), biopesticides (eg. Bacteria, fungi) and organic compounds (eg. Organophosphate pesticides (OPP), carbamates). Among organic pesticides, there are several classes: organochlorine pesticides (OCP) (eg. DDT group), phenol derivatives, carbamates, aryloxy-carboxylic acid derivatives, OPP (eg. phosphoric esters (dichlorvos)), organometallic, alkaloids, quaternary ammonium salts (eg. Paraquat, diquat, chlormequat). OCPs and OPPs (malathion, parathion, diazinon, chlorpyrifos) and carbamates (carbaryl, carbofuran, aldicarb) are insecticides used to control pests in agricultural food commodities.

Foodstuffs of plant origin can be contaminated by pesticide residues. Moreover, pesticide residues can remain for years in soil and water [2]. Food-producing animals can eat or drink contaminated products [3,4]. Therefore they can enter in the food chain.

The risks to human health from exposure to pesticides range from acute to chronic low-dose toxicity [5,6]. The toxic effects of pesticides can be different with risk factors depending on the exposure and the pesticide: local irritation (eg. Skin or ocular irritation), respiratory syndrome, allergic reactions, neurodevelopmental effects caused by enzyme inhibition (ie. Cholinesterase (OPP, carbamates) [7], neurologic issues (eg. OCP), and oxidative damages.

Regulation 396/2005 established maximum residue limits (MRLs) for permitted pesticides in or on foodstuffs intended for human or animal consumption. These MRL were set on the basis of

compliance with good agricultural practice and guaranteed consumer safety [8]. The control of pesticides is ensured through annual monitoring programs (NRMP) that requires Member States to sample, analyse and test an agreed range of products for pesticides to ensure compliance with pesticide MRLs and to assess consumer exposure. The most commonly used methods for the analysis of pesticides are chromatographic methods (gas chromatography and liquid chromatography coupled to mass spectrometry in tandem (GC-MS/MS, LC-MS/MS)) [9,10].

A pesticide detection method must have several characteristics, similar to those required for clinical point of care tests (POCT), which are very well defined by the acronym ASSURED. The ASSURED criteria defined by World Health Organization (WHO) means Affordable, Sensitive, Specific, User friendly, Rapid and Robust, Equipment-free and Deliverable to end users [11,12]. Biosensors for the detection of pesticides in food products are able to offer these performance characteristics [13]. Therefore biosensors represent a promising avenue for pesticide detection [14–16]. It has been explored since the 80's [17–19].

The detection of pesticide residues can be performed using different recognition elements: antibodies (immunosensors) [20], molecularly imprinted polymers (MIPs) [21,22], aptamers [23,24], bacteria (Stoytcheva 2011) or yeast (Schofield 2007), enzymes [25,26], and more recently nanomaterials that have enzyme-like properties [27–29]. Regarding the transduction elements, mainly electrochemical [30–35] and optical biosensors [36–38] have been developed. In this review, we will focus on enzymatic colorimetric and dual-ratiometric biosensors.

This review will look at the different sensing principles of enzymatic colorimetric sensors, and will present illustrated examples of applications in water, plant and animal-derived food. Numerous colorimetric and dual ratiometric biosensors based on enzymatic inhibition have been developed for the detection of OPPs and carbamates in water and in various matrices of plant origin (ie. fruits, vegetables). Different types of substrates (ie. chemical, polymer and nanomaterials based) and/or fluorophores will be presented for colorimetric and fluorimetric detection. These biosensors could reach very low detection limits (down to ng/ml or ng/l). However, these methods are not necessarily specific to pesticides, since other chemical agents can inhibit acetylcholinesterase-type enzymes (eg Sarin,). This review will highlight that in recent years, nanomaterials have been increasingly used to improve detection sensitivity.

2. Principle of enzymatic biosensors for pesticide analysis

Enzymatic biosensors for pesticide detection were mostly based on the inhibition of the enzyme activity by the pesticide. The pesticide took the place of the enzyme substrate and thus inhibited the activity of the enzyme. **Figure 1** presented the structures of the main pesticides used as targets in AChE-based biosensors [39].

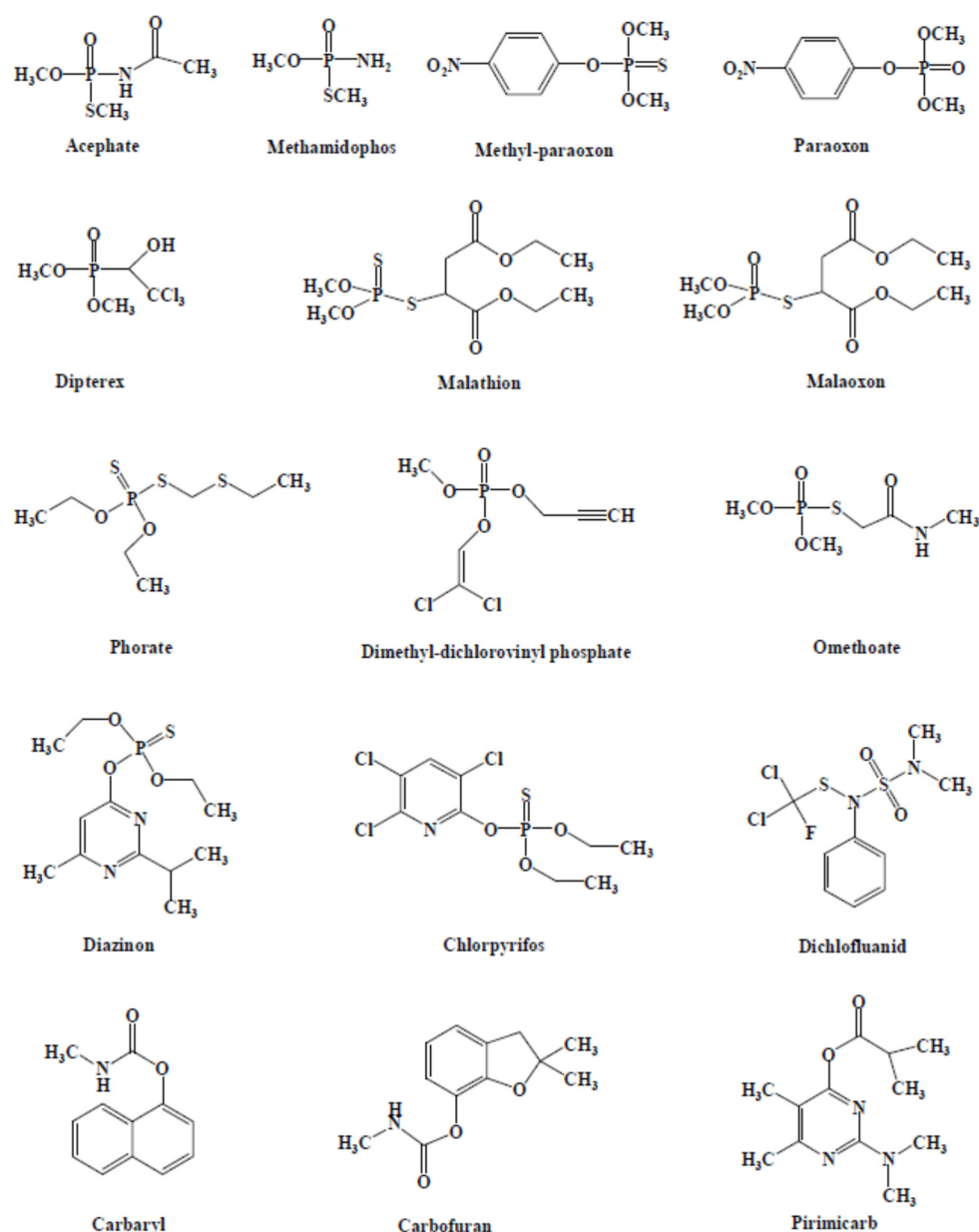


Figure 1. Structures of the main pesticides used as targets in AChE-based biosensors [39] (This article is licensed under an open access Creative Commons CC BY 4.0 license).

The different enzymes used for the development of enzymatic biosensors for the detection of pesticides were: acetylcholinesterase (AChE) [40], butyrylcholinesterase (BChE)[41], choline oxidase (ChO) [42], organophosphorus hydrolase (OPH) [43], alkaline phosphatase (ALP) [44], tyrosinase (TYR) [45] and laccase (LAC) [46].

Whatever the enzyme, the substrate bound to the active site of the enzyme. The substrate was then converted into a product which was released into the environment and left room for a new substrate molecule to bind to the enzyme (**Figure 2**).

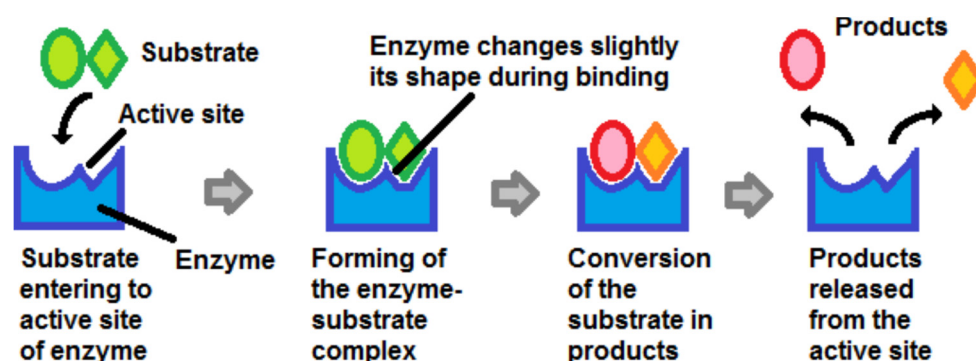


Figure 2. Description of the catalytic activity process of an enzyme [47] (Creative Commons Attribution 3.0 Unported License).

This review will mainly focus on AChE because it was the mostly used enzyme. However, some examples of sensors based on ChO, and ALP will also be discussed.

Acetylcholinesterase (AChE, EC 3.1.1.7) catalyzed the rapid hydrolysis of the neurotransmitter acetylcholine into acetate and choline (**Figure 3**). Organophosphorus pesticides (OPPs) and carbamates had an inhibitory activity on enzymes such as AChE and BChE (EC 3.1.1.8) which made them potentially biocidal and toxic. This inhibition was due to the blocking of the esteratic site of the enzyme (phosphorylation or carbamylation of the OH of the serine of the active site) by the pesticide, which competed with the specific substrate of the enzyme (ie. Acetylcholine). Therefore the acetylcholine was no more transformed into choline. Generally the cation-binding site (ie. Anionic site) did not interact with OPP. Their inhibitory action led to an accumulation of acetylcholine at the level of synapses, resulting in over-activation of cholinergic receptors and cholinergic toxicity (e.g. involuntary movements, muscle paralysis, bradycardia, dyspnea). Therefore cholinesterases could be used as a recognition tool for the detection of pesticides.

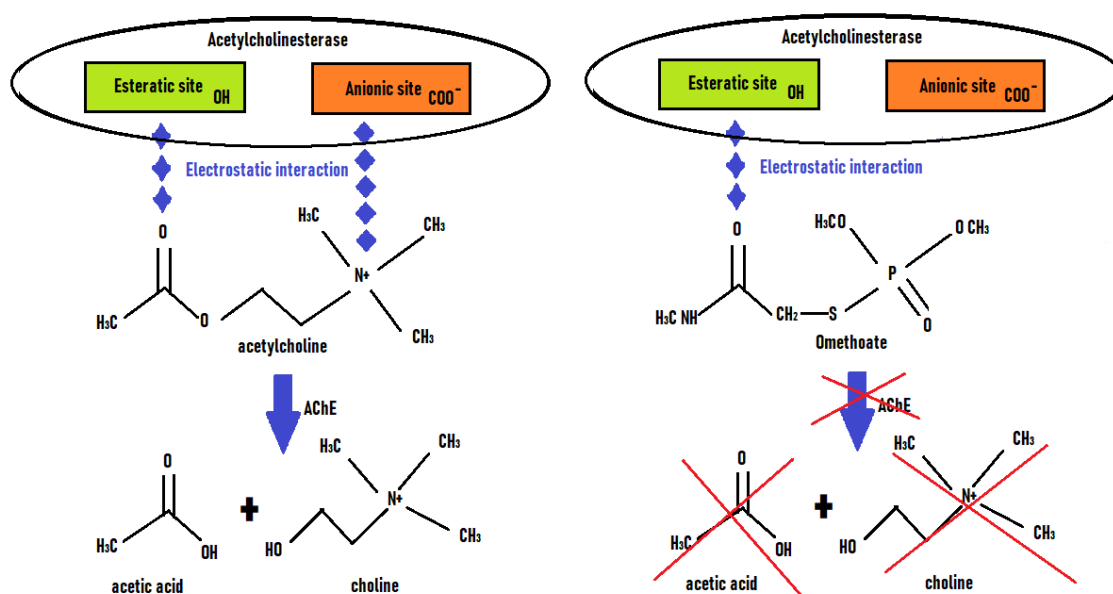


Figure 3. Schematic representation of the active sites of the enzyme acetylcholinesterase involved in the catalytic activity and its inhibition by an organophosphorous pesticide. Acetylcholine interacted electrostatically with the enzyme which led to the production of acetic acid and choline. In the presence of an inhibitor (ie. Omethate) the esteratic site interacted electrostatically with omethate. Therefore the catalytic activity was inhibited with no production of acetic acid nor choline.

The production of recombinant AChE could be a solution to develop more sensitive and selective biosensors for the detection of pesticides. This was due to a better knowledge and understanding of the interaction and binding of the inhibitor with the active site of the enzyme [48,49].

In the presence of ChOx (EC 1.1.3.17), following the catalytic activity of AChE, choline was oxydated to betaine and released hydrogen peroxide (H_2O_2). Then H_2O_2 could oxidise a chemical reagent (eg. Dye 2,20-azino-di-[3-ethylbenzthiazoline sulfonate (ABTS) [42], 3,3',5,5'-tetramethylbenzidine (TMB) [50]) that produced a change of colour or fluorescence.

In the presence of the enzyme ALP (3.1.3.1), the substrate p-aminophenyl phosphate (p-APP) could be hydrolyzed to p-aminophenol (p-AP). The enzymatic sensor was usually based on the reduction of a chemical element by p-AP (ie. Reduction of manganese dioxide (MnO_2) to Mn^{2+} [51]). When the enzymatic activity was inhibited by a pesticide, the product p-AP was not formed so that the reduction did not occur.

The immobilisation of the enzyme was an essential step to maintain its structural integrity and therefore its function. It is necessary to guarantee the stability of the biosensor but also to allow access of the substrate and the inhibitor to the active site of the enzyme (ie. Relevant orientation of the enzyme) in order to obtain optimal sensitivity and specificity. Different immobilisation techniques were available: adsorption, covalent binding, entrapment, encapsulation, cross-linking [52]. The cost of immobilisation partly related to the choice of the support materials should also be taken into account in the development of field biosensors.

3. Colorimetric enzymatic biosensors

The first colorimetric methods were developed to detect acetylcholinesterase activity [53,54]. Then development of colorimetric biosensors using AChE for the detection of inhibitors (ie. Pesticides, neurotoxic agents) started in the years 1990's [36,55].

Basically the principle of the colorimetric detection of an enzymatic inhibitor was as follows. Without an inhibitor, enzymatic activity took place and so the product was synthesised by the catalytic activity of the enzyme. The formed product could react with a colorimetric substrate (eg. Dye precursor, nanoparticles) which produced a change of colour (by oxidation or reduction). In the presence of an inhibitor, the enzyme did not convert the substrate into the product. Thus the product could not perform the colorimetric detection mechanism.

There were different types of reagents used for the colorimetric detection of inhibition of the enzymatic activity of cholinesterases or related enzymes. They could be classified into three categories: chemical, polymer and nanomaterials based. **Table 1** shows examples of colorimetric enzyme-based biosensors developed for the detection of organophosphate pesticides and carbamates.

3.1. Chemical based

Chemical based enzymatic sensors use classical chemical reagents for the colorimetric detection of the enzymatic product.

Table 1. Different colorimetric enzymatic sensors for the detection of pesticides.

Substrate/method	Pesticide	Enzyme	Construction of the sensor/Sensing principle	Matrices	LOD	Reference
Ellman	Chlorpyrifos	AChE	pSBMA-μPAD on cellulose filter + DTNB	Apple, orange, spinach, tomato, cucumber	0.235 mg/l	[56]
	7 types of OPP	AChE	Zeolitic imidazolate framework (ZIF)-8 for AChE encapsulation + DTNB (in solution)	Extract of Brassica rapa	Glyphosate, glufosinate, malathion 10 ⁻⁵ ppb LOQ 0.05, 0.01, and 0.05 ppb	[57]
	Paraoxon	AChE	Biopolymer membranes for AChE immobilisation (cross-linking GA)+ DTNB (96-wells plate)	/	10 ng/ml	[58]
	Malathion	AChE	Eggshell membrane (cross-linking GA)+ DTNB (in solution)	/	0.1 ng/ml	[59]
	Omethoate, Methidathion Malaoxon	AChE	AChE immobilised by adsorption + DTNB (microplates, in solution)	Olive oil	1.3.10 ⁻⁶ (0.3 ppm), 2.4.10 ⁻⁸ (0.002 ppm) and 4.10 ⁻⁹ M (0.001 ppm)	[60]
	Methomyl, profenofos	AChE	AChE immobilised on biopolymer chitosan gel by crosslinking GA + DTNB (test strip)	Water	6.16 × 10 ⁻⁴ mM 0.27 mM	[61]
	Malathion	AChE	AChE immobilised by adsorption + DTNB Colorimetric strip	/	2.5 μg/ml	[62]
IDA	Chlorpyrifos	AChE	AChE immobilised by adsorption + IDA Colorimetric strip Smartphone reading	Cabbage	3.3 μg/ml	[63]
	Chlorpyrifos	AChE	AChE immobilised by adsorption + IDA Colorimetric strip	/	8.60 ppm (μg/ml)	[64]
	Paraoxon and trichlorfon	AChE	AChE immobilised by adsorption + IDA Colorimetric strip	Apple juice	Scanner: 0.01 ng/ml and 0.04 ng/ml Visual reading: 0.03 ng/ml and 0.1 ng/ml	[65]
	Pyridostigmine, tacrine, paraoxon, carbofuran, soman and VX	AChE	AChE immobilised by adsorption + IDA Colorimetric strip	/	10-100 nM	[66]
	Paraoxon (OPP)	AChE	AChE immobilised by adsorption + IDA Colorimetric strip	Water	10 ⁻⁷ M	[67]

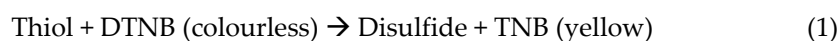
	Dipterex	AChE	Multilayer paper chip with IDA and AChE Colorimetric strip (μ PAD)	/	0.0406 mg/l	[68]
IPA/IDA	Parathion, chlorpyrifos and malathion	AChE	AChE immobilised by adsorption + IDA or IPA Colorimetric strip	Rice and lettuce	10^{-3} μ g/ml	[69]
IPA	Bendiocarb Carbaryl Paraoxon Malathion	AChE	AChE and IPA entrapped into polyvinyl amine (PVAm) Colorimetric strip	Milk and apple juice	~1nM ~10 nM ~1 nM ~10 nM	[70]
Others	Methyl paraoxon	ChOx	Oxidation of ABTS dye by H_2O_2 ($\lambda = 734$ nm)	Water	58 μ M	[42]
	Carbaryl, chlorpyrifos	AChE	BCP (pH indicator) Colorimetric strip	Water	0.24, 2 μ g/l	[71]
	OPPs	AChE	Bromothymol blue Colorimetric strip	Tea	0.0001 ppm à 0.1 ppm	[72]
TMB	Trichlorfon	AChE	TCh => Inhibition of TMB oxidation COF nanozyme (TpBTD) => Oxidation of TMB	Apple, broccoli, cucumber	0.11 ng/ml (spectrophotometer) 1.29 ng/ml (smartphone)	[73]
	Methyl parathion	AChE	TCh => aggregation of AuNPs AuNPs catalyzed the oxidation of TMB.	Water	0.21 ng/ml	[74]
	Carbaryl	AChE	Double strand DNA-SYBR green I complex => oxidation of TMB	River water	0.008 ng/ml	[75]
	Paraoxon	AChE and ChOx	TCh => Reduction of TMB ACh => H_2O_2 => decomposition of MnO_2 NF => oxidation of TMB blocked	Apple-banana juice	0.5 ng/ml	[50]
	Parathion ethyl	AChE	Without inhibitor: Choline + C-AuNPs => TMB not oxidized (colourless)	/	5.8 ng/mL	[76]
	Methyl parathion	AChE and ChO	Inhibitor: Monomers of C-AuNPs (nanozyme) => TMB oxidised => blue colour (652 nm) CoOOH NFs => TMB oxidised (blue) with the help of H_2O_2		10 ng/ml	[77]
	Dichlorvos	AChE	PolyPCDA/lipid derivatives + myristoylcholine => colour change. Hydrolysis of myristoylcholine by AChE => no change	/	6.7 ppb	[78]
Nanomaterials	Malathion	ALP	AuNP@ MnO_2 NPs p-AP => reducing agent of MnO_2 NPs to Mn^{2+}	Apple and lettuce	0.82 pg/ml	[51]

Chlorpyrifos, methyl, omethoate, bromophos-ethyl	AChE	TCh => reducing agent of MnO ₂ NPs to Mn ²⁺	Grape juice, leaves (corn, rice, peanuts)	0.5 µg/l	[79]
Malathion	AChE	TCh => Aggregation of AgNPs on GO leaves => decrease in the SPR band intensity	Water and grapes	0.01 pM	[80]
Omethoate	ALP	ALP-induced silver metallisation (Ag-Au nanorods) => colour change	Grapes juice	83.2 ng/l	[81]
Chlorpyrifos	AChE	TCh => aggregation of GQD-AuNPs => colour change 3D-µPAD	Vegetables	131 nM 0.0007 µg/ml	[82]
Paraoxon, parathion, fenitrothionand diazinon,	AChE	TCh => aggregation of AuNPs => colour change	Water	0.13 ng/ml, 0.37 ng/ml, 0.42 ng/ml and 0.20 ng/ml	[83]
Parathion	AChE	TCh => reducing agent of Au ³⁺ and protect the AuNPs from dissolution by the Au ³⁺ -CTAB	Tap water, apple washing solution, and sea water	0.7 ng/ml	[84]
Methamidophos malathion	ALP	Enzyme-stimulated silver deposition Au@Ag NPs (λ = 370 nm)	Water	0.025 µg/l 0.036 µg/l	[85]
Methyl-paraoxon chlorpyrifos-oxon	AChE + ChO	Nanoceria-coated device => yellow colour in the presence of H ₂ O ₂ (ChO product)	Cabbage and dried seafood (mussels)	18 ng/ml 5.3 ng/ml	[86]
Paraoxon	AChE	TCh => aggregation of LA-AuNPs => colour change	Apple juice	4.52.10 ⁴ pM	[87]

ABTS: 2,20-azino-di-[3-ethylbenzthiazoline sulfonate]; AChE: acetylcholinesterase; AgNPs : silver nanoparticles; ALP: alkaline phosphatase; ATCh: acetylthiocholine iodide; Au³⁺-CTAB: Au³⁺-cetyltrimethylammonium bromide; AuNPs : gold nanoparticles; BCP: Purple Bromocresol; ChOx: choline oxidase; COF: Covalent organic framework; CoOOH NFs: cobalt oxyhydroxide nanoflakes; GQD-AuNPs: graphene-quantum-dot capped gold-nanocomposite particles; LA: lipoic acid; LSPR: localized surface plasmon resonance; µPAD: microfluidic paper-based analytical devices; MnO₂ NPs: manganese dioxide nanoparticles; NWs: nanowires; p-AP: p-aminophenol; pSBMA: poly(sulfobetaine methacrylate); PdSP@rGO: Palladium square nanoplates on reduced graphene oxide; polyPCDA : poly(10,12-pentacosadynoic acid); RB: rhodamine B; TCh : thiocholine ; TMB: 3,3',5,5'-tetramethylbenzidine; TpBTD: 2,4,6-triformylphloroglucinol (Tp) and 4,4'-(benzo[c][1,2,5]thiadiazole-4,7-diyl) dianiline (BTD); TPPS1 : monosulfonate tetraphenyl porphyrin; ZIF-8 : zeolitic imidazolate framework.

3.1.1. Ellman

The Ellman's method was a classical colorimetric method used for the detection of thiol groups [54]. The mechanism of action of the Ellmann reagent was as follows [88]. Thiols reacted with Ellmann's reagent, (5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (faintly coloured) cleaving the disulphide bond to give 5-nitro-2-thiobenzoic acid (TNB) (yellow colour) in water at neutral and alkaline pH visible at 412 nm.



The Ellman method thus found application for the determination of cholinesterase (ChE) activity using thiocholine esters as substrates [54,89]. This method was applicable to acetylcholinesterase [90] and butyrylcholinesterase detection [91]. When thiocholine was produced by the catalytic activity of ChE, this product owing a thiol group could react with DTNB to form the yellow product TNB (**Figure 4**). In the presence of an inhibitor, thiocholine was no more produced. Therefore DTNB was not transformed in TNB, the solution remained colourless. To measure cholinesterase activity accurately and precisely, the rate of hydrolysis of the substrate by the enzyme should be slower than the Ellman reaction between thiol groups and DTNB.

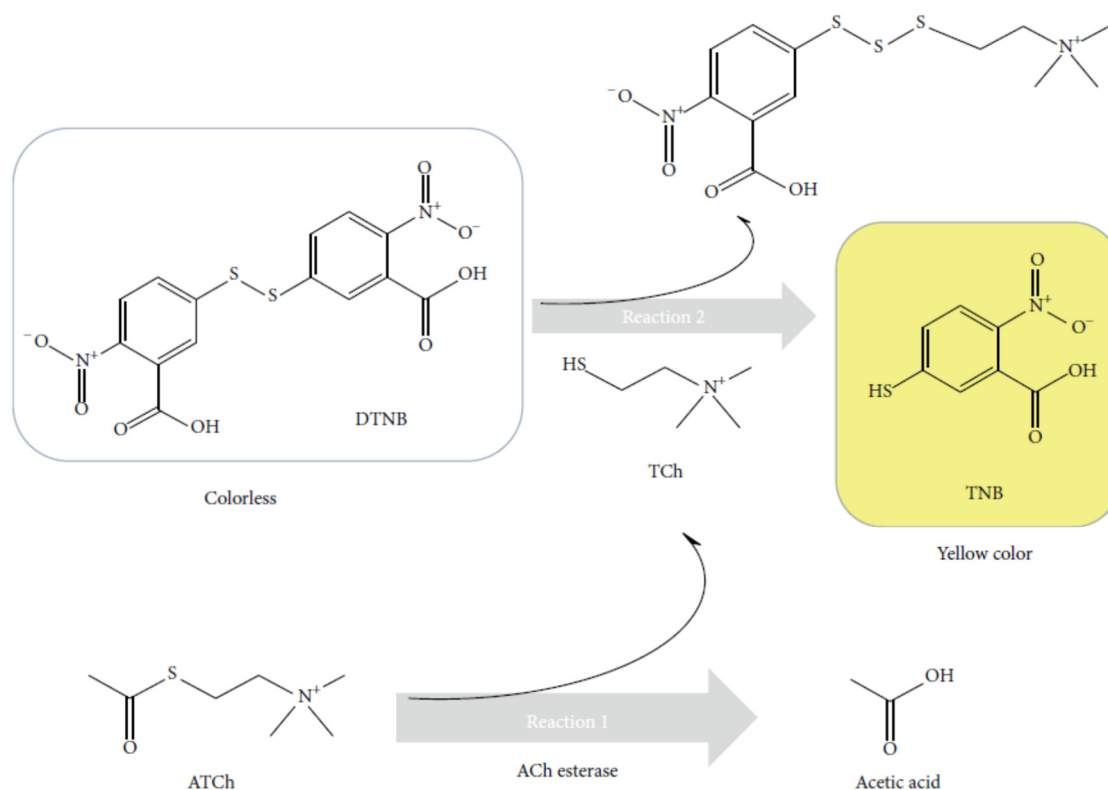


Figure 4. Mechanism of action of the Ellman reagent for the detection of acetylcholinesterase activity [62] (This is an open access article distributed under the Creative Commons Attribution License Attribution 3.0 Unported (CC BY 3.0)).

Cacciotti et al. developed a multiplex-based 96-well system for the detection of malathion [58]. Three different biopolymers were tested for the immobilisation of AChE (i.e. polylactic acid (PLA), polycaprolactone (PCL), and poly-hydroxybutyrate-co-hydroxyvalerate (PHBV)). Biopolymeric electrospun fibrous materials are ecodesigned supports. Two methods of immobilisation were compared, physisorption and cross-linking with glutaraldehyde. Finally the biopolymer PHBV was selected as the best support to immobilise AChE by crosslinking method. The detection limit for paraoxon was 10 ng/ml. Kaur et al. developed an AChE biosensor for the detection of malathion [59].

They also used the immobilisation of the enzyme onto the eggshell membrane by cross-linking with glutaraldehyde. The detection limit was reported at 0.1 ng/ml.

One of the currently used support material to build colorimetric biosensors was paper (ie. Cellulose). Indeed it was a low-cost and easy to use platform for the development of diagnostic tests [92]. Paper support was biomolecules friendly. Immobilisation of enzymes was simple and non-selective adsorption was limited. The paper allowed the development of microfluidic systems, lateral flow assays that could allow the separation of different components. Examples of paper-based colorimetric sensors will be presented below. The colorimetric paper-based sensor developed by Kavruk et al. was not enough sensitive, with a detection limit at 2.5 $\mu\text{g/ml}$ [62]. The enzyme was physically adsorbed on paper filter with the substrate and some stabilisers (sugar (eg. Glucose) or protein (bovine serum albumine (BSA)) to improve the biosensor shelf-life.

A team developed a paper-based sensor comparing different enzymes (AChE and BChE) and different support materials indicator (glass and cellulose) [93]. This method used the Ellman's reagent to detect the production of thiocholine. The fabrication of the sensor is presented in **Figure 5**. All the reagents were deposited on the paper-based sensor on two different places (**Figure 5a**). They were put in contact by folding the strip (**Figure 5b**). The yellow colour was visible on the strip when the sample did not contain inhibitors. The comparative results between the two enzymes and the two different supports (glass and cellulose) were presented in **Figure 5c**. The inhibition caused by the presence of phytostigmine was clearly measured in all cases. The best results were obtained with the enzyme AChE (LOD 0.0005 $\mu\text{g/ml}$).

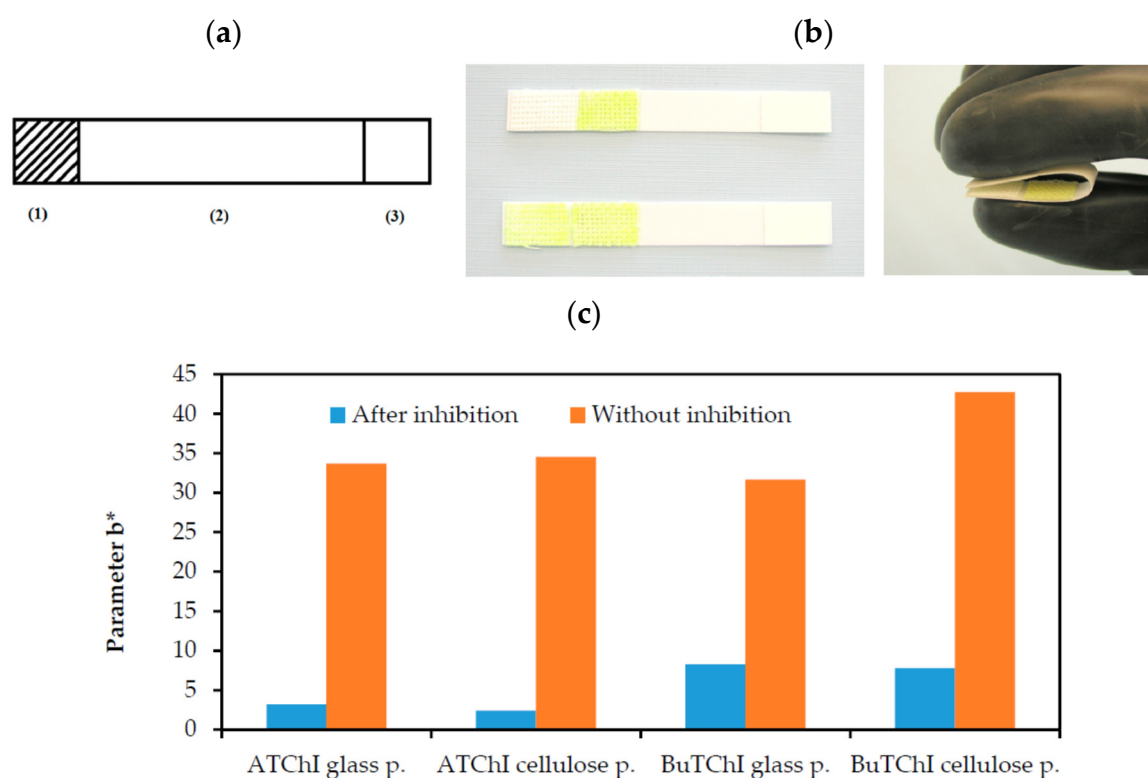


Figure 5. Development of a cellulose filter paper-based acetylcholinesterase sensor using Ellman's reagent as the indicator [93]. A Scheme of the prepared sensor ((1) immobilised enzyme, (2) plastic strip, (3) substrate and indicator) ; B Sensor before and after inhibition test; C Results of inhibition test with phytostigmine on different supports (glass and cellulose) and with two different enzymes (parameter b is the analytical signal). (This article is licensed under an open access Creative Commons CC BY 4.0 license).

Metal Organic Frameworks (MOFs) are crystalline porous nanomaterials constituted of self-assembly of inorganic metal centers (metal ions or metal clusters) (eg.iron, zinc, nickel, copper,

zirconium, ...) and bridging organic ligands (eg. imidazolate) [94]. The interesting properties of MOFs that could be exploited to develop sensitive enzymatic biosensors are their high surface area, porosity, and the reversible encapsulation/release of guest molecules like enzymes. The encapsulation of enzymes into MOFs could increase their stability, and enhance their catalytic activity. Kukkar et al. developed an acetylcholinesterase-based biosensor where the enzyme was encapsulated into zeolitic imidazolate framework (ZIF)-8 to produce a nanoreactor AChE@ZIF-8 NR (**Figure 6a**) [57]. They showed that the biosensor combining AChE and ZIF-8 showed a 10-fold increase in biocatalytic activities compared to the native AChE-based biosensor. The substrate DTNB was used for the colorimetric determination ($\lambda = 412$ nm) using UV-visible spectrophotometer. The change of colour in relation to the different glyphosate concentrations from 0.01 to 100 ng/ml could be visible by naked eyes (**Figure 6b**). This biosensor was able to detect seven different OPPs. The LOD for three pesticides (glyphosate, glufosinate and malathion) were estimated almost identically as 10^{-5} ng/ml. This colorimetric biosensor was developed in solution. Their perspective was to develop a paper-based biosensor strip based on the same principle.

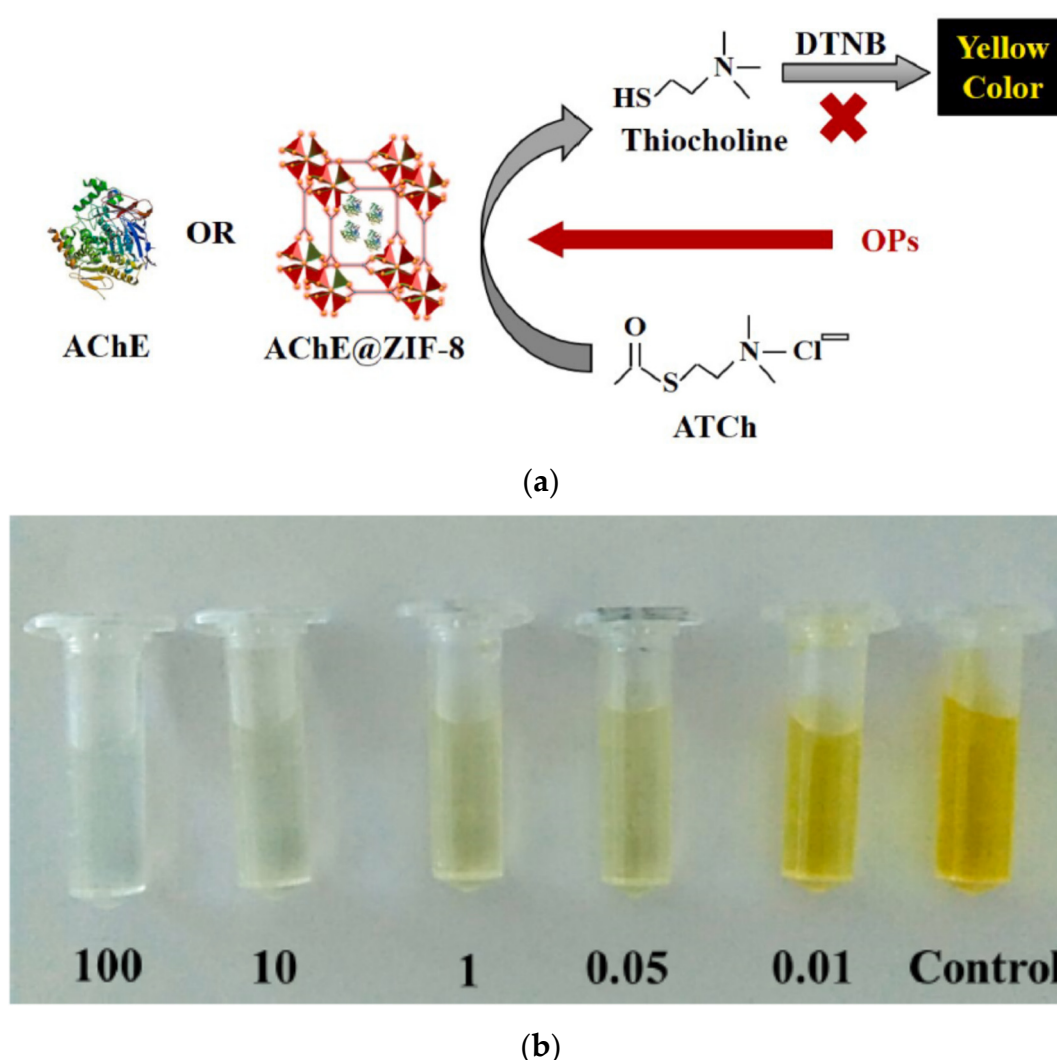


Figure 6. (a) Schematic principle of colorimetric detection of different glyphosate concentrations using enzymatic nanoreactor built on zeolitic imidazolate framework-8 (ZIF-8). (b) Visual detection of different glyphosate concentrations from 0.01 to 100 ng/ml. Reprinted with the permission from [57]. Copyright © 2021 Elsevier.

The Ellman's method faced some drawbacks such as the low stability of DTNB and the existence of interferences with substances containing thiol groups (eg. Amino acids (cysteine), reduced glutathione) [95].

3.1.2. Indoxyl acetate (IDA), 2,6-dichloroindophenol acetate (DCIP) and indophenyl acetate (IPA)

The substrate indoxyl acetate (IDA) was a fluorogenic as well as chromogenic reagent acting also as a substrate of AChE and butyrylcholinesterase. Indeed IDA was hydrolysed in the presence of the enzyme AChE and H₂O to form a single indoxyl radical and acetic acid (**Figure 7**). Then two single indoxyl radicals reacted together to form at the end (2,2'-Biindoline)-3,3'-dione (Indigo, blue). The change of colour from colourless to blue could be detected optically at 670 nm.

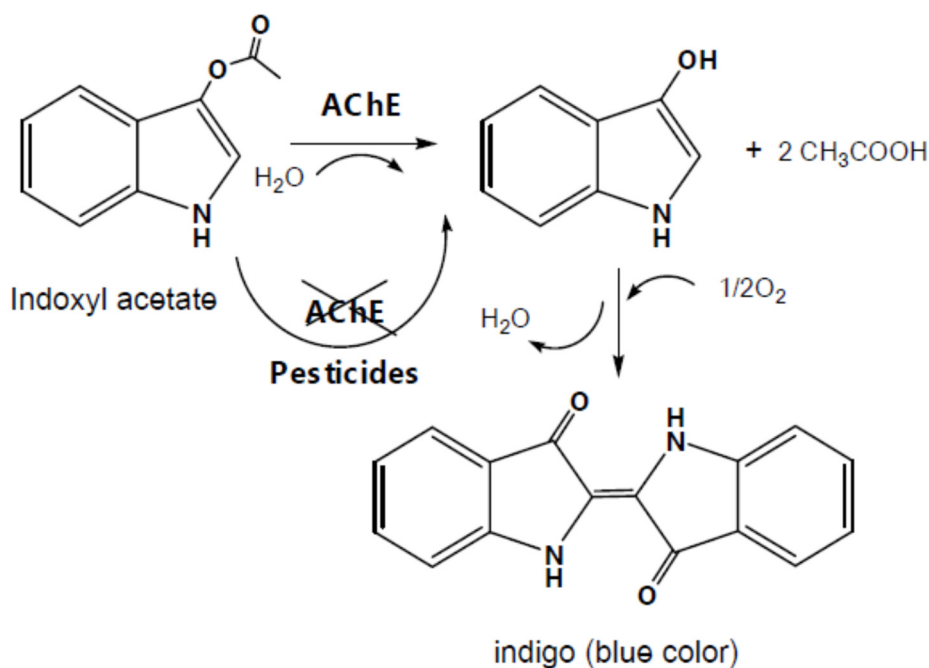


Figure 7. Mechanism of action of indoxyl acetate as AChE chromogenic substrate [96] (This is an Open Access article distributed under the terms of the Creative Commons Attribution Licence (<http://creativecommons.org/licenses/by/4.0/>)).

Pohanka et al. proposed an alternative chromogenic substrate to DTNB, the 2,6-dichloroindophenol acetate (DCIP), a blue redox dye ($\lambda = 606$ nm) [95,97]. In acidic medium, the blue DCIP (oxidised) was transformed into 2,6-dichloroindophenol that is colourless (reduced). They reported that no interferences with reduced glutathione and albumin were observed in the tested conditions. However ascorbic acid can interact with the detection because DCIP was initially used for the detection of ascorbic acid (ie. reducing agent). The highest drawback of this method was that the reaction is much longer than Ellman's method because of a low turnover rate. The advantage of DCIP was that the change of colour is more intense than the one of Ellman's reagent, so easier to measure.

Pohanka et al compared three different substrates (DTNB, DCIP and IDA) to develop a biosensor for the detection of pesticides, based on BuChE (**Figure 8**) [97]. DCIP was reported as the best substrate because the lowest LOD was obtained and the biosensor was less sensitive to potential interferences.

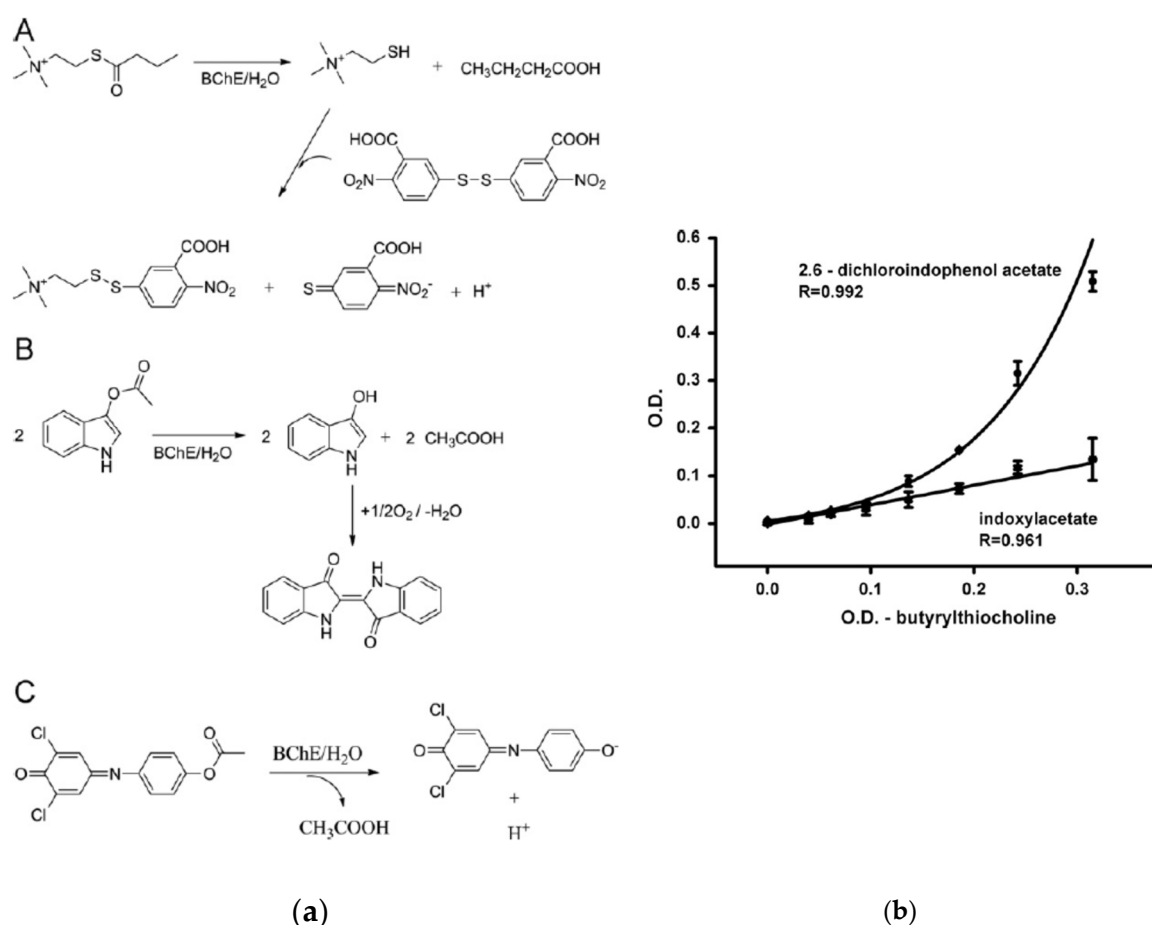


Figure 8. (a) Comparison of three different substrates for butyrylcholinesterase: (A) DTNB; (B) indoxylacetate (IDA), (C) 2,6-dichloroindophenol acetate (DCIP). (b) Correlation of the methods on standard 96 wells microplates. Reprinted with permission from [97]. Copyright © 2013 Elsevier.

The colorimetric reading could be performed visually, with a classical spectrophotometer or more recently with a smartphone. Reading with smartphone is more and more developed for field applications (ie. water quality monitoring [98]). Smartphone reading could be based on smartphone applications [74,99] or softwares [77] that could interpret the images taken by the smartphone.

While classical spectrophotometer are benchtop equipment, the main advantages of smartphone reading are simplicity and portability. Digital camera based analytical devices like smartphones are widely used in everyday life, used and usable by everyone, at reasonable cost. Finally this technology is easily applicable to paper-based biosensors, lateral-flow immunoassays (LFIA) and other types of biosensors. Therefore smartphone reading are really a promising tool for hand-held assays, especially for monitoring pesticide residues in food products.

Indophenyl acetate (IPA) was used for the determination of AChE activity since the 1950's. The substrate IPA was hydrolyzed by AChE at pH 8.0. The change of colour was from red-yellow to blue purple [53]. This simple method was optimised (eg. Substrate concentration, pH, time before absorbance measurement) and was reliable to measure between 25 to 150 units of acetylcholinesterase. More recently, some colorimetric biosensors based on AChE were developed based on IPA. For instance, Fu et al. developed a colorimetric biosensor based on a dipsticks and a smartphone reader for the detection of chlorpyrifos in cabbage [63]. A colorimetric dipstick was produced, with two sides, one containing AChE and one the substrate IPA, that folded together to bring the reagents into contact, after the sample has been deposited (Figure 9). The change of colour from red (indophenol acetate) to blue (indigo), was measured with a digital camera and a smartphone. The transmission light intensity was measured by the smartphone and the signal was interpreted by a free application. A LOD of 3.3 µg/ml was reported.

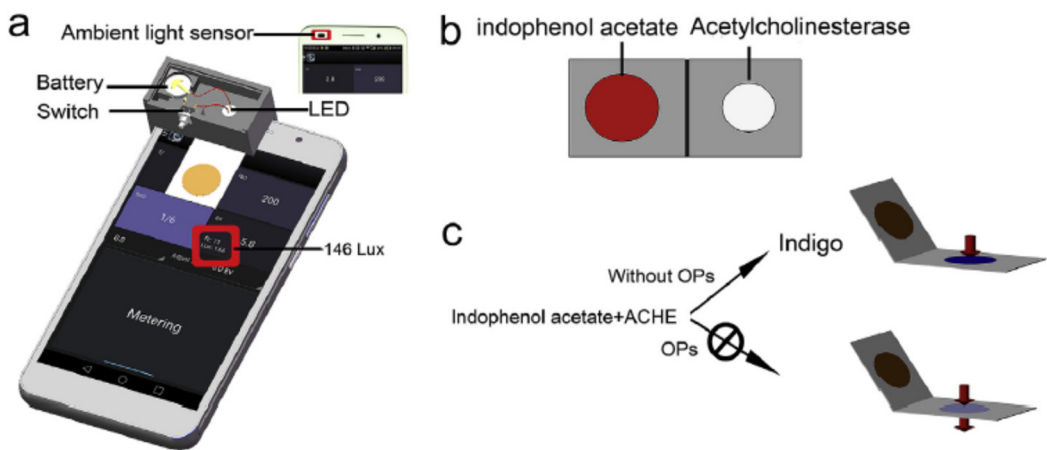


Figure 9. Colorimetric test strip based on AChE and indophenol acetate for the detection of chlorpyrifos in cabbage [63]. Reprinted with the permission from [63]. Copyright © 2019 Elsevier.

Nowadays, many smartphone readers used RGB principles for the colorimetric reading. RGB is an optical colour processing system, trichromy based on the three primary colours red, green and blue (RGB). A value between 0 and 1 is assigned to each of the 3 primary colours to define a colour that makes up an image.

Pohanka et al. developed a biosensor for the detection of AChE and BuChE activity in plasma samples with a smartphone reading based on RGB principle [100]. The substrate was deposited onto multiwell 3D-printed pad (Figure 10). Then a smartphone (LED light included) was placed onto the pad, and a photograph in .jpg format was taken. A software was used to determine the colour depth of each well. The R value equal to 0 is the darkest red, while the R value 255 is the brightest red. They concluded that indoxylacetate provided a change in colour depth more than three times higher than that of 2,6-dichlorophenolindophenyl acetate. Amongst the three channels, the R channel appeared to be the most sensitive regarding the calibration curves, showing the lowest LOD. The readings were performed in parallel with a classical spectrophotometer to validate the smartphone readings. They observed no significant difference between BuChE activity determined by spectrophotometric method and smartphone reading.

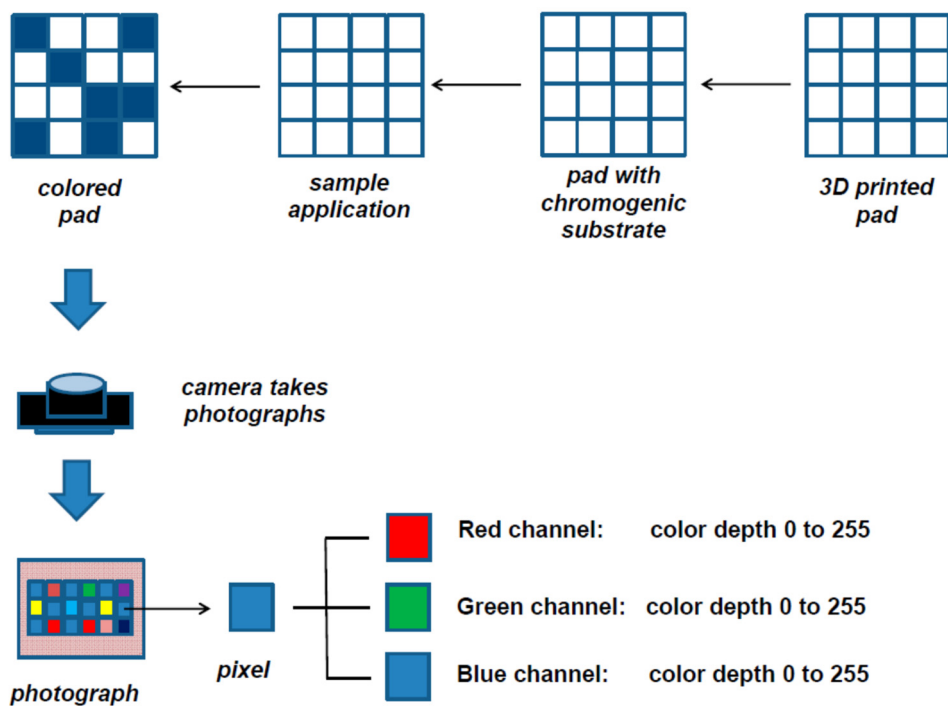


Figure 10. Principle of a biosensor for the detection of AChE and BuChE activity in plasma samples based on multiwell 3D-printed pads and smartphone reading based on RGB principle [100] (This article is licensed under an open access Creative Commons CC BY 4.0 license).

3.1.3. Hestrin

The Hestrin method was an alternative to the Ellman method for visualising AChE inhibition [101]. The main difference between the two methods was that the analytical signal depended on the concentration of the substrate (acetylcholine chloride) in the Hestrin method, whereas it depended on the concentration of the reaction product in the Ellman method. In the Hestrin method, acetylcholine was determined by its reaction with hydroxylamine at an alkaline pH, in the presence of ferrous ions. To our knowledge, this method was very little implemented to develop enzymatic biosensors for the detection of pesticides. This method was applied once to the monitoring of blood cholinesterase activity in workers exposed to organophosphate pesticides (nerve agents) [102]. The conclusion was that the results obtained with this method were not very reproducible or valid.

3.1.4. TMB

The Horseradish peroxidase (HRP) enzyme catalysed the hydrolysis of substrates in the presence of hydrogen peroxide (H_2O_2). The substrate 3,3',5,5'-tetramethylbenzidine (TMB) was the most currently used chromogenic substrate for the enzyme HRP. TMB was a colourless or pale blue-green substrate. After the action of HRP, TMB took on a blue colour whose absorbance could be measured between 620 and 655 nm.

A first alternative to HRP was based on the acetylcholine (ACh) itself mimicked peroxidase's activity. A colorimetric system containing ACh, TMB and H_2O_2 was developed for the sensitive and selective determination of AChE activity and detection of its inhibitors. Therefore due to the catalysis of ACh by AChE, the peroxidase-like activity was affected and the signal decreased. This phenomenon was exploited for the detection of AChE activity with a LOD of 0.5 mU/ml for AChE activity and 4 ng/ml for OPP detection [103].

A second alternative to HRP is to exploit the enzyme-like activity of some nanomaterials, called nanozymes, in combination with TMB. The catalytic properties of MnO_2 nanoparticles were frequently used for the development of colorimetric sensors. For instance, a colorimetric biosensor based on AChE was developed based on the oxidation power of manganese dioxide (MnO_2) nanosheets onto the substrate TMB (ie. mimic oxidase activity) [104]. Indeed thiocholine (TCh) was able to reduce the oxidation power of MnO_2 nanosheets. Therefore less oxTMB were produced that resulted in decrease in the absorbance ($\lambda = 652$ nm) (ie. light blue to colourless). In the presence of an inhibitor, less TCh was generated. As a result, the blue colour was stronger and the absorbance increased. The combination of MnO_2 nanoflakes (NFs) and TMB was also used to develop a colorimetric bi-enzymatic sensor. Jin et al. developed a multienzyme-hydrogel sensor based [50]. Firstly AChE catalyzed the hydrolysis of ACh and then choline is oxidised by choline oxidase (ChO) to produce H_2O_2 . As a consequence, MnO_2 NFs lost their oxidation power and the oxidation of TMB was blocked. In the presence of an inhibitor, the production of H_2O_2 was reduced and MnO_2 nanoflakes recovered their oxidation power and so oxTMB was produced (strong blue). The detection limit obtained for paraoxon was of 0.5 ng/ml. The sensor was able to detect 3 μ g/ml of paraoxon in apple-banana juices by naked eyes reading. Jin et al. developed a multi-enzyme colorimetric biosensor for the detection of parathion-methyl based on the peroxidase-mimick activity of cobalt oxyhydroxide nanoflakes ($CoOOH$ NFs) which catalyzed TMB into blue oxTMB with the help of H_2O_2 [77]. Again the bi-enzymatic system was constituted of AChE and ChO. This biosensor was able to detect parathion methyl as low as 10 ng/ml.

A colorimetric assay based on the combination Ag [I] ion-TMB was developed for the detection of inhibitors of AChE [105]. Ag [I] ions were able to oxidise TMB (oxTMB (deep blue)). When AChE was introduced, TCh produced by the enzymatic reaction reacted with Ag [I] ions. Therefore Ag [I] ions could not oxidise TMB and a pale blue colour was observed (reduced TMB (colourless)) (**Figure 11**). In the presence of an inhibitor, the amount of TCh decreased and therefore the reaction with Ag

[I] ions also decreased. Ag [I] ions could oxidize TMB and the colour was then be a deeper blue (oxTMB).

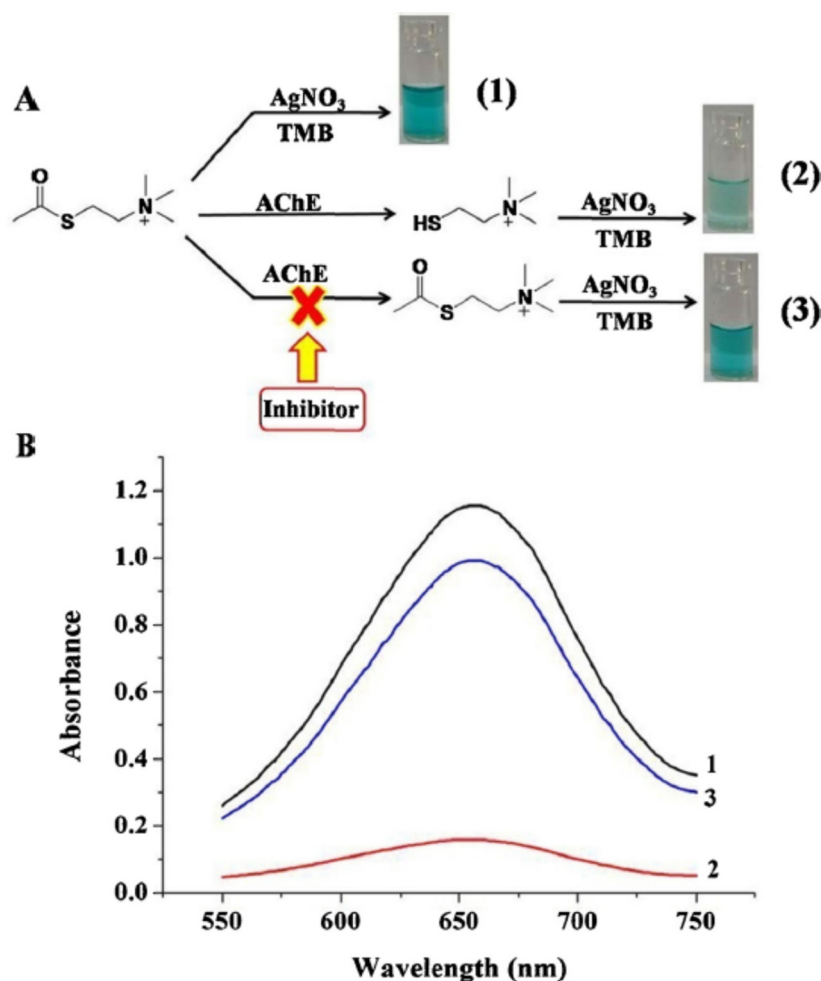


Figure 11. Colorimetric acetylcholinesterase-based sensor [105]. Thiocholine interacts with Ag [I] ions and reduces oxTMB to reduced TMB (colourless). In the presence of an inhibitor, the amount of thiocholine decreases. Therefore Ag [I] ions are free to oxidize TMB and the colour will then be a deeper blue (oxTMB). B. The three curves correspond to Ag [I] ions in the presence of TMB and curve 1. 10 μM ATCl; curve 2 10 μM ATCl and AChE; curve 3 10 μM ATCl, AChE and the inhibitor neostigmine. Reprinted with the permission from [105]. Copyright © 2016 Elsevier.

Gold nanoparticles (AuNPs) were frequently used for the development of colorimetric sensors, especially in combination with TMB. Liu et al. developed an acetylcholinesterase assay for the detection of parathion methyl in water [74]. When not aggregated, AuNPs could oxidise TMB. In the presence of TCh, the aggregation of AuNPs was induced and TMB remained not oxidised (colourless) (Figure 12). When a pesticide inhibited the enzymatic activity, TMB could be oxidised by AuNPs because TCh is not produced. This biosensor showed a detection limit of 0.21 ng/ml.

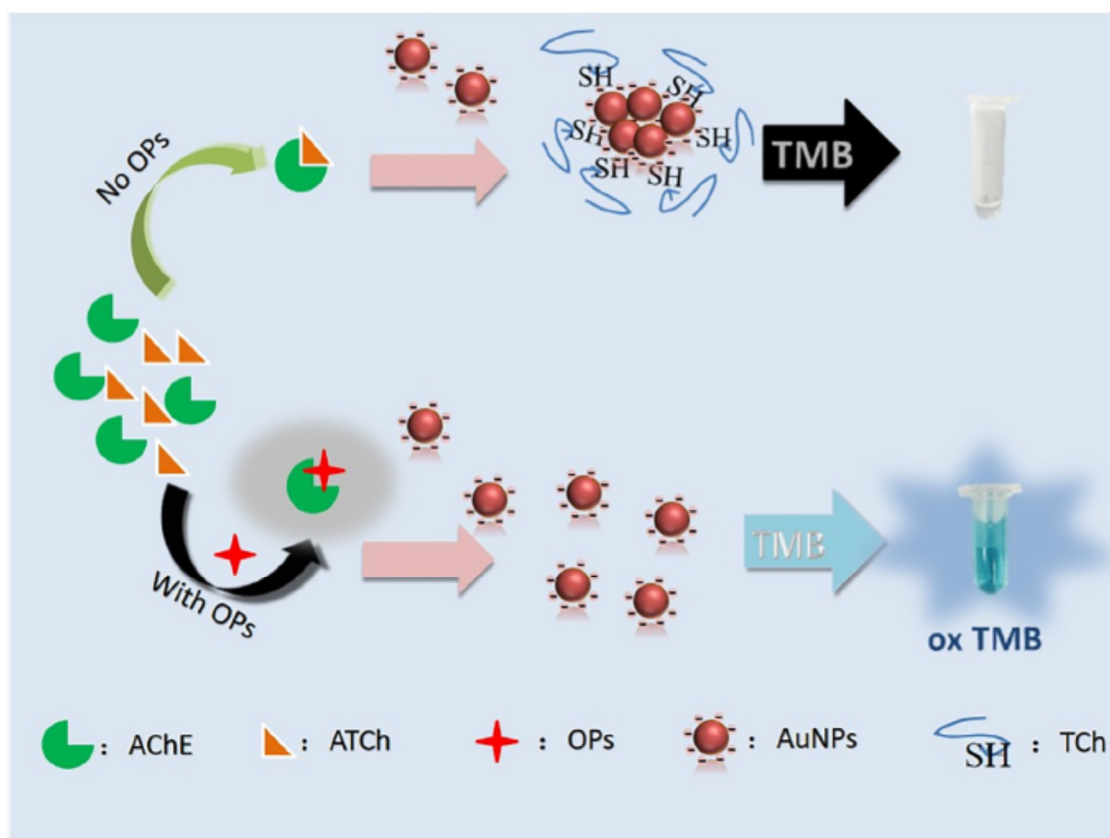


Figure 12. Principle of a colorimetric gold nanoparticle-based acetylcholinesterase assay for the detection of parathion methyl [74]. Reprinted with permission from [74]. Copyright © 2022 Elsevier.

Negatively charged cysteamine capped gold nanoparticles (C-AuNPs) were used to develop an AChE based colorimetric sensor for the detection of parathion ethyl [76]. The peroxidase-like activity of C-AuNPs was exploited in combination with colorimetric detection with TMB. In the presence of AChE, choline reacted with C-AuNPs, which prevents the oxidation of TMB (colourless) (**Figure 13**). In the presence of parathion ethyl, lesser choline was produced, the non aggregated C-AuNPs could oxidise TMB which gave a deep blue colour. The increase in the absorption at 652 nm was directly proportional to the concentration of pesticide. The LOD was reported at 5.8 ng/ml.

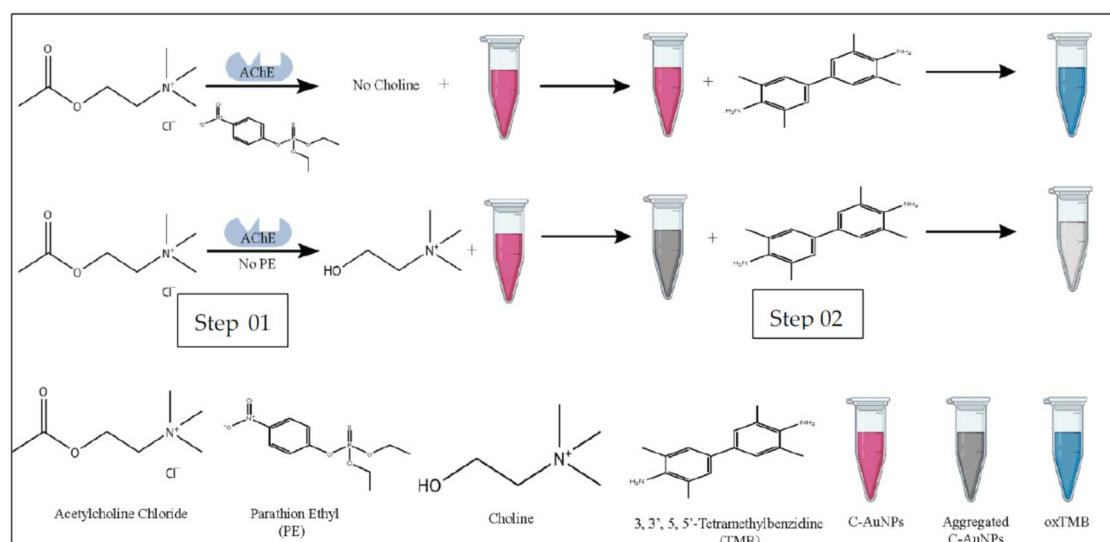


Figure 13. Colorimetric detection of parathion ethyl using 3,3',5,5'-tetra-methylbenzidine (TMB) combined with cysteamine capped gold nanoparticles (C-AuNPs) [76]. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

3.1.5. Others

The dye bromothymol blue was used to develop an AChE paper based colorimetric sensor for the detection of ofatox in tea [72]. Test cards were prepared by spraying AChE directly onto paper filters, as well as enzyme substrate and bromothymol blue onto three different paper filters. Then the three papers were stacked together and pressed between two metallised PET film sheets. A hole is performed onto the test card to drop the sample. The LOD of the test card for Ofatox in tea was 0.1 ppm ($\mu\text{g/ml}$).

Another chromogenic substrate was used for the detection of pesticides, using the inhibition of another enzyme, ChOx (**Figure 14**) [42]. ChOx oxidised choline to betaine and H_2O_2 . In the presence of the enzyme HRP and the generated H_2O_2 , a dye, 2,20-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) was oxidised. Then the absorbance increased at 734 nm and the dye changed from colourless to blue-green. In the presence of a pesticide, paraoxon methyl, the enzymatic activity of ChOx was inhibited which reduced the colour change and consequently the absorbance. The LOD was reported at 58 μM .

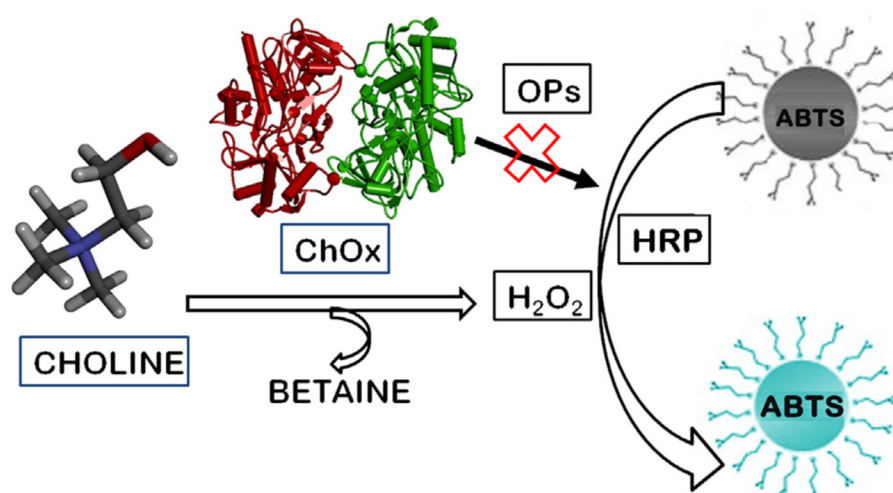


Figure 14. Schematic principle of the colorimetric enzymatic sensor for the detection of pesticides. Reprinted with permission from [42]. Copyright © 2022 Elsevier.

Polymers or nanofibres are often considered as promising alternative materials that are widely applied for colorimetric and fluorescence detection. Poly(10,12-pentacosadynoic acid) (poly-PCDA) vesicles were used to develop a colorimetric enzymatic biosensor based on the induction of the polymer by myristylcholine, which was one of the substrates of AChE [78]. It was hypothesized that cationic surfactants like esters of choline could induce the colour transition of poly-PCDA. Indeed in the presence of myristylcholine, the colour transition of the polymer from blue to red induced by myristylcholine occurred (**Figure 15**). Therefore when there is no pesticide, choline is produced, less myristylcholine is available; so the colour transition did not occur or decreased. In the presence of the pesticide, choline was not or less produced, more myristylcholine remained. Therefore the colour transition occurred. When poly-PCDA was used without lipids, the observation by naked eyes could only detect dichlorvos at 150 ppb (ng/ml) which is not enough sensitive. Therefore they produced some poly-PCDA vesicles by mixing the polymer with different lipids. The lipid dimiristoylphosphatidyl-choline (DMPC) gave the best results with a LOD for dichlorvos of 6.7 ng/ml. Furthermore they were able to detect 50 ng/ml of dichlorvos by naked eyes with poly-PCDA vesicles which is three times more sensitive than with poly-PCDA alone.

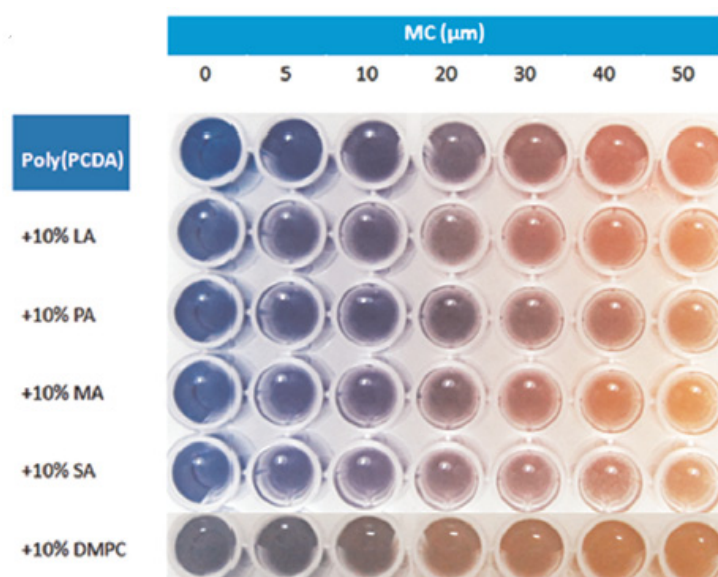


Figure 15. A. Colour development of poly-PCDA with different concentrations of myristylcholine (MC) and different lipids. Reprinted from permission from [78]. Copyright © 2014 Elsevier.

3.2. Nanomaterials based

3.2.1. Gold and silver nanoparticles

The following optical properties of gold nanoparticles were very interesting for the development of colorimetric biosensors: good optical characteristics, high electron densities, good biocompatibility and catalytic performance [106]. The change of colour between aggregated and disaggregated state of NPs was exploited to develop colorimetric biosensors, without the needs of other colorimetric substrates. In solution, dispersed AgNPs were yellow in colour, upon aggregation the nanoparticles appeared orange/red. Dispersed AuNPs in solution were red in colour, upon aggregation the nanoparticles appeared blue. AgNPs extinction coefficients were higher than those of AuNPs of the same average size [107]. Yet AuNPs were more commonly used because the functionalisation of AgNPs often led to their degradation; AuNPs were more stable. Furthermore colour changes of AuNPs could be easily observed visually compared to AgNPs [107].

Some chemical substances (eg. Pesticides, herbicides, metallic ions) could prevent the aggregation of AgNPs and AuNPs. **Figure 16** shows how these analytes could interact with the gold and silver nanoparticles [108]. The nanoparticles could aggregate in the presence of inducing agents. In the presence of the analyte, an anti-aggregation effect of the analyte occurred. Three different principles are illustrated: i) the anti-aggregation effect was caused by the replacing of the inducing agent by the analyte to the NPs, due to a repulsing effect of the inducing agent; (ii) the anti-aggregation effect occurred because the analyte promoted the redox reaction of aggregation inducing agent and (iii) the anti-aggregation effect was due to the complexation of the analyte with the aggregation inducing agent.

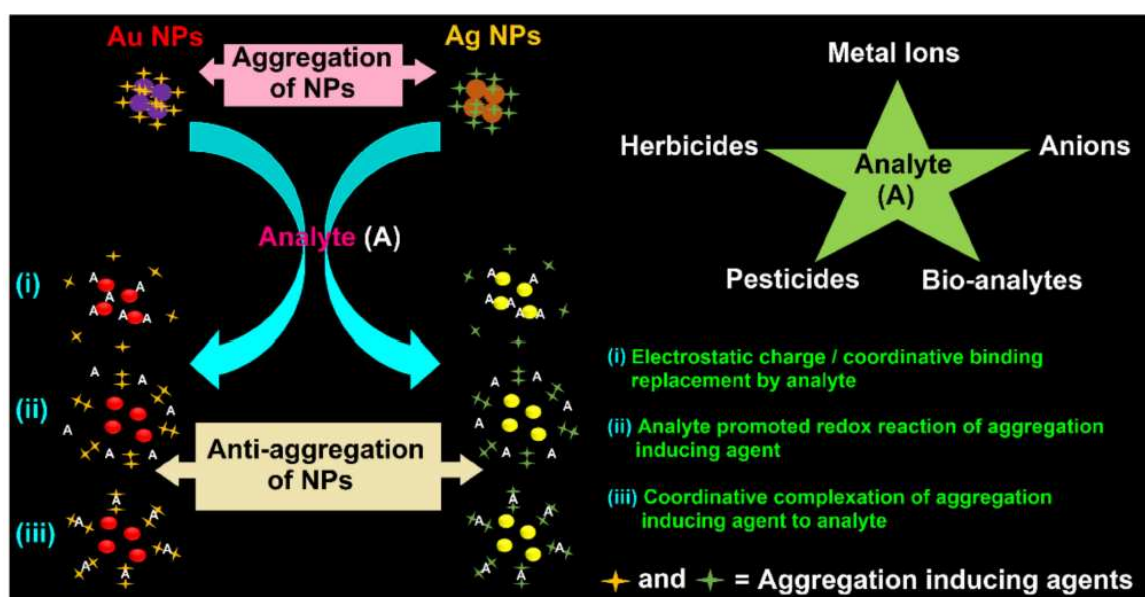


Figure 16. Colorimetric sensors based on the anti-aggregation of gold and silver nanoparticles by chemical substances [108]. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

For instance, thiocholine could induce the aggregation of AgNPs and AuNPs. A colorimetric sensor based on AChE and graphene quantum dots capped gold-nanocomposite particles (GQD-AuNPs) for the detection of chlorpyrifos was developed [82]. The three-dimensional microfluidic paper-based analytical device (3D- μ PAD) reached a very low LOD (0.0007 μ g/ml). Another colorimetric sensor was based on the aggregation of AgNPs on graphene oxide (GO) leaves in the presence of TCh [80]. A LOD as low as 0.01 pM was reported for malathion in water and grapes.

Three different ways could be used to induce the aggregation of gold nanoparticles: addition of capping agent on the surface of AuNPs (e.g. thiols, amines, phosphines), surface functionalization (e.g. macromolecules, polymers) and addition of salt (e.g. NaCl) [107]. High salt concentrations result in the aggregation of AuNPs. They can be used to enhance the stability of colloid AuNPs.

A colorimetric AChE-based biosensor was developed using citrate capped AuNPs for the detection of several OPP in water samples (Figure 17) [83]. Gold nanoparticles were aggregated when TCh was produced by the catalytic activity of AChE. Indeed positively charged TCh was attracted by the negatively charged citrate groups located on the surface of AuNPs. On the contrary, AuNPs were dispersed in the presence of the pesticide because thiocholine was not produced. The lowest LOD of 0.13 ng/ml was reported for paraoxon in water.

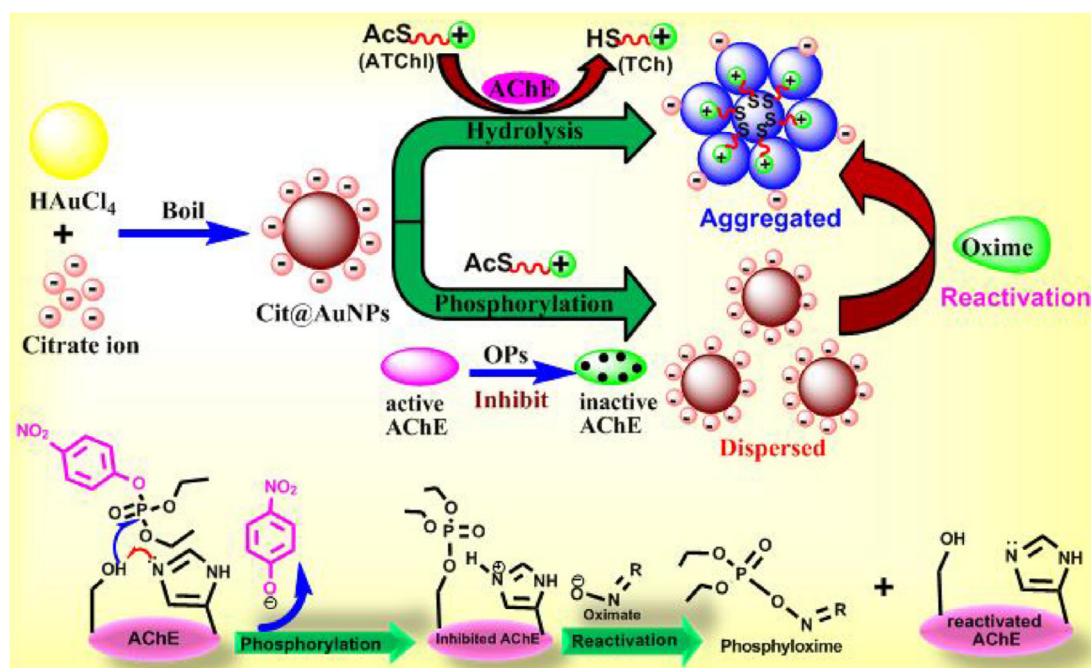


Figure 17. Principle of an acetylcholinesterase based colorimetric biosensor using citrate-capped gold nanoparticles. Reprinted with the permission from [83]. Copyright © 2018 Elsevier.

The aggregation potential of lipoic acid capped gold nanoparticles was used to develop a colorimetric sensor for the detection of paraoxon in apple juice (**Figure 18**) [87]. Thiocholine could induce the aggregation of LA capped AuNPs along with a distinct colour change from red to steel-blue. In the presence of a pesticide, TCh was no more produced, so that AuNPs could not aggregate anymore and the colour did not change. The reported LOD of $4.52 \cdot 10^4$ pM was very low.

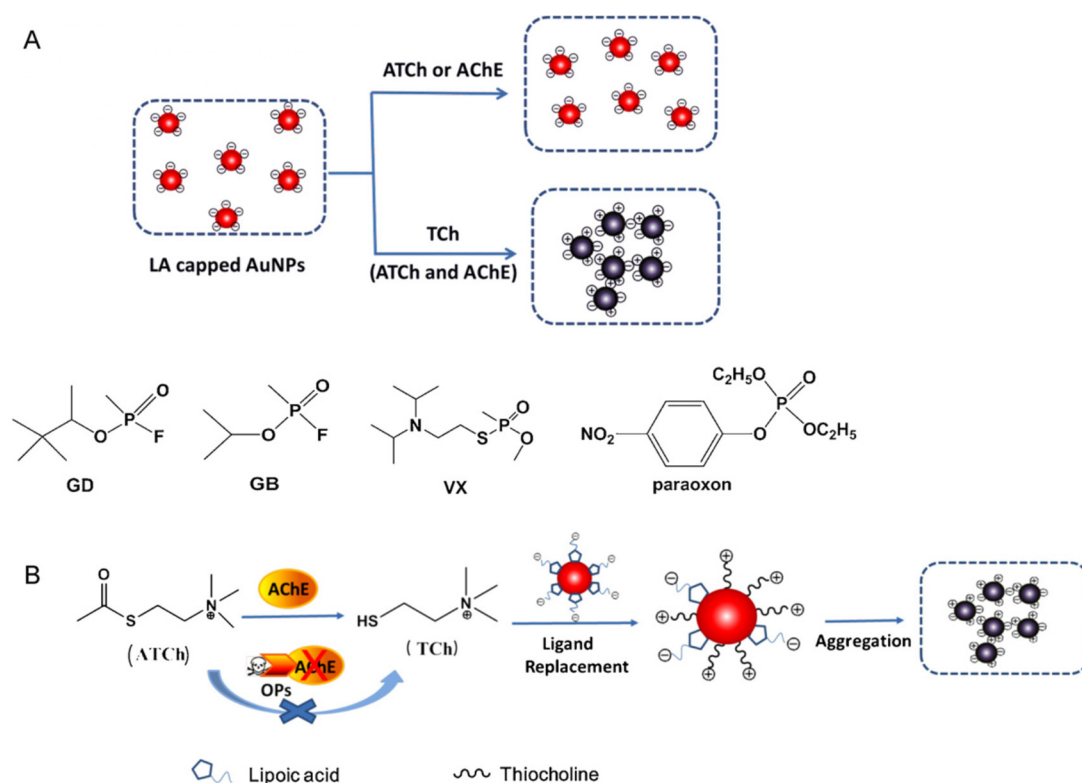


Figure 18. Lipoic acid capped AuNPs-based colorimetric detection of paraoxon. Reprinted with the permission from [87]. Copyright © 2011 Elsevier.

Localized surface plasmon resonance (LSPR) is an optical phenomena generated by light when it interacts with conductive nanoparticles (NPs) [109]. The adsorption of molecules (ie. pesticides) to the surface of nanoparticles caused surface plasmon resonance changes that could be monitored. It was observed that wavelength shifts of the LSPR were accompanied by colour changes that could be measured also. **Figure 19** showed the colour changes in relation (a) to the different tested pesticides and (b) to the wavelength shifts for the same pesticides [110]. It was clear that the biosensor did not have the same detection sensitivity for all the pesticides tested. The best sensitivity was obtained for ethion and the worst for paraoxon ethyl.

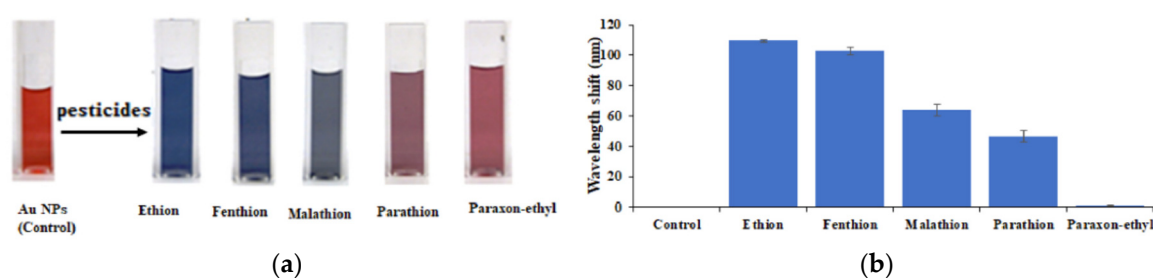


Figure 19. (a) Colour changes and (b) shifts in LSPR for AuNPs in the presence of different organophosphate pesticides. Reprinted from permission from [110]. Copyright © 2019 Elsevier.

Lv et al. developed an enzymatic biosensor based on a combination of ALP with gold and silver NPs [85]. p-aminophenol (p-AP), the product of the catalytic activity of the ALP enzyme, was able to reduce Ag(I) ion to Ag metal that was spontaneously deposited on the surface of AuNPs to form Au@Ag NPs. The formation of Au@Ag NPs led to a change of colour, from red to yellow first, and then to gray. This change of colour could be detected visually or by spectrophotometry. The LOD were reported as 0.025 $\mu\text{g/l}$ and 0.036 $\mu\text{g/l}$ for methamidophos and malathion respectively.

A colorimetric biosensor was developed based on the inhibition of the ALP-induced silver metallization on the surface of gold nanorods (Au NRs) in the presence of omethoate (**Figure 20**) [81]. The silver ions were reduced by ascorbic acid (AA), the product of the dephosphorylation of acid-2 phosphate (AAP) due to the catalytic activity of ALP. Then silver nanoshells were deposited onto the surface of AuNRs to form Ag@AuNRs. It resulted in a series of blue shifts on the LSPR peak. In the presence of omethoate, the catalytic activity of ALP was inhibited. Therefore silver ions were not reduced and so AuNRs were not modified. Rainbow-like multicolour changes from pink to green to cyan to violet to yellow-red could be observed that were correlated to the concentration of omethoate. Indeed the colour changes from the infrared to the visible range were related to the changes in the size, shape, and composition of Au NRs, in relation to the extent of Ag-shell deposition on the Au NRs. The actual changes of colour in the microwells in relation to the omethoate concentrations (from 20 to 10000 $\mu\text{g/l}$) could be visible by naked eyes (**Figure 21a**). The wavelength changes in the UV-vis spectra were visible in relation to the omethoate concentration (**Figure 21b**). The wavelengths difference were proportional to the concentration of omethoate and showed a good linear relationship (**Figure 21c**). It was possible to semi-quantify the concentration of pesticide, even by naked eyes. The detection limit obtained with a microplate reader was low (83.2 ng/l).

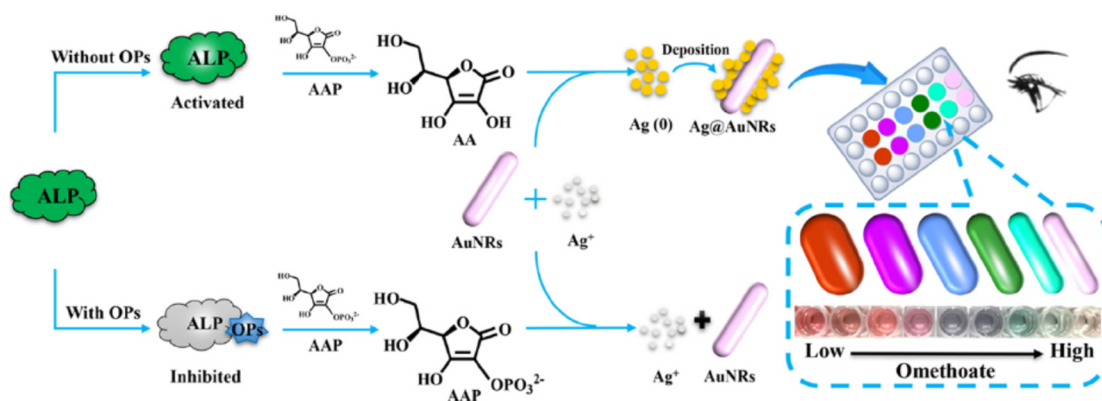
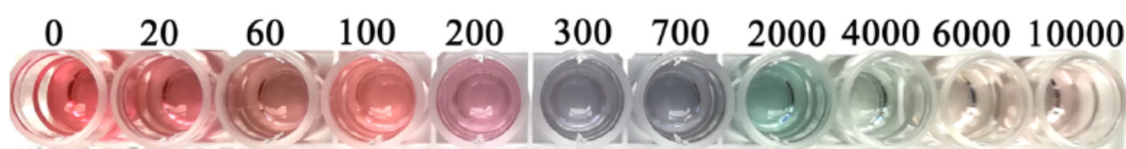
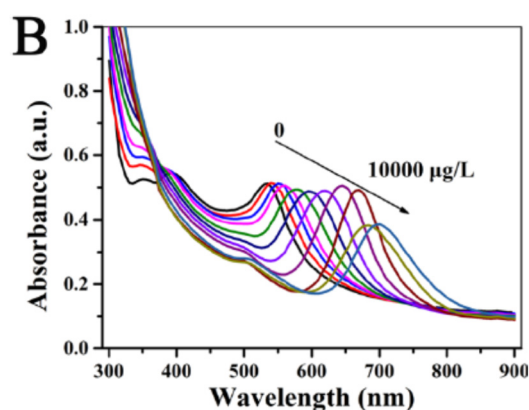


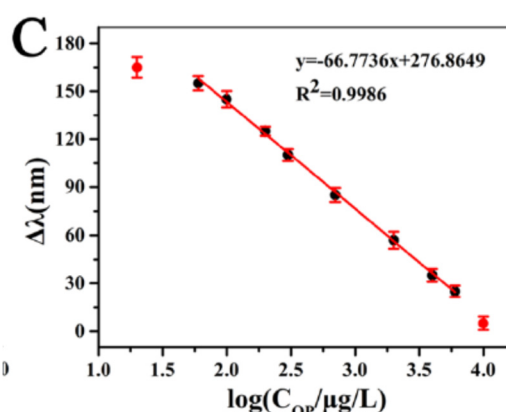
Figure 20. Schematic principle of a multicolorimetric biosensor based on the inhibition of the ALP-induced silver metallization on the surface of gold nanorods (Au NRs) in the presence of omethoate. Reprinted with permission from [81]. Copyright © 2020 American Chemical Society (ACS).



(a)



(b)



(c)

Figure 21. Multicolorimetric biosensor based on the inhibition of the ALP-induced silver metallization on the surface of gold nanorods (Au NRs) in the presence of omethoate: (a) Changes of colour in the microwells in relation to the omethoate concentrations (from 20 to 10000 µg/l) could be visible by naked eyes; (b) UV-vis spectra of a designed multicolor colorimetric sensor for different omethoate concentrations; (c) The wavelengths difference were proportional to the concentration of omethoate. Reprinted with permission from [81]. Copyright © 2020 American Chemical Society (ACS).

A gold nanoparticles-based paper colorimetric biosensor was developed for the detection of organophosphorous pesticides [84]. This biosensor was based on the enzymatic hydrolysis reaction of acetylcholinesterase and gold nanoparticles (AuNPs) in Au^{3+} -cetyltrimethylammonium bromide (Au^{3+} -CTAB) solution-coated papers (**Figure 22**). The property of Au^{3+} to dissolve the AuNPs was used, thus leading to a visible colour change. However when thiocholine was produced by AChE activity, it reduced Au^{3+} , preventing the dissolution of AuNPs and the change of colour. The role of CTAB was to stabilise the AuNPs and to prevent them from aggregation in high salinity conditions. The LOD obtained with the AuNPs-coated dipstick was 35 ng/ml for parathion.

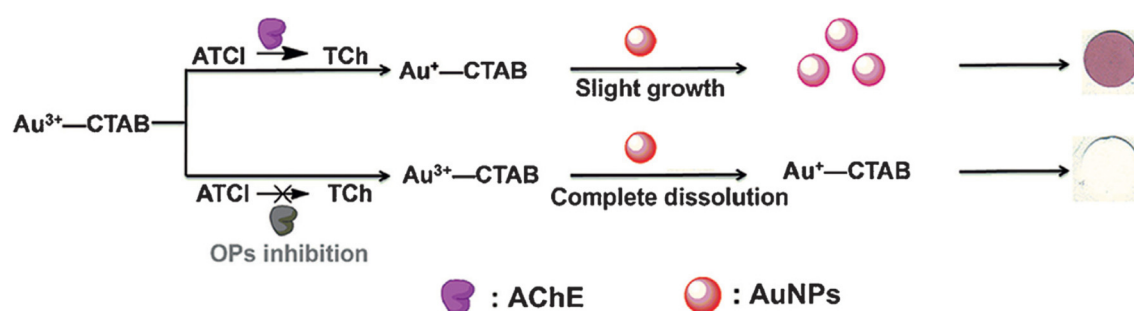


Figure 22. Principle of a AuNPs-based paper colorimetric biosensor for the detection of organophosphorous pesticides based on the enzymatic hydrolysis reaction of acetylcholinesterase and gold nanoparticles (AuNPs) in Au³⁺-cetyltrimethylammonium bromide (Au³⁺-CTAB) solution-coated papers. Reprinted with permission from [84]. Copyright © 2017 Elsevier.

3.2.2. Other nanoparticles

Manganese oxide nanoparticles (MnO₂) could be also used for the development of colorimetric biosensors. Qian et al. developed a biosensor based on MnO₂ NPs, UFO-shaped Au@MnO₂ nanosheets and AChE inhibition by different OPP (chlorpyrifos, methyl, omethoate, bromophos-ethyl) [79]. Thiocholine is a reducing agent for NPs. The colour of MnO₂ NPs changes from dark brown to light brown, while the colour of Au@MnO₂ nanosheets changes from green to pink. The LOD was 0.5 µg/l. The sensor was applied to the detection of OPP to grape juice and leaves (corn, rice, peanuts). Huang et al. developed an enzymatic biosensor based on alkaline phosphatase and a combination of gold and manganese oxide nanoparticles (ie. MnO₂-coated gold nanoparticles (GNP@MnO₂ NPs)) [51]. p-aminophenol reduced the MnO₂ NPs to Mn²⁺ ions, which broke the core-shell nanostructure GNP@MnO₂ NPs; thus the gold core is exposed to light. The colour changed from bright yellow (GNP@MnO₂ NPs) to green (gold core). In the presence of malathion, the ALP activity was inhibited; thus the color change did not occur or more lightly. A very low LOD of 0.82 pg/ml for malathion was reported. This method was applicable to actual samples of apple and lettuce.

Cerium oxide nanoparticles (Nanoceria) are metal oxide nanoparticles, oxidase-like properties in aqueous environments [111]. Nuanthavong et al. developed a nanoceria-based biosensor for the detection of methyl-paraoxon and chlorpyrifos-oxon [86]. The principle of the paper-based analytical device (PAD) was summarised in **Figure 23** [112]. In the presence of acetylcholine, H₂O₂ was produced by the bi-enzymatic system (AChE and ChO). Thus the colour changed from colorless to yellow when nanoceria were in the presence of H₂O₂. When AChE activity is inhibited by pesticides, less H₂O₂ was produced, then the PAD remained colorless or with less intense yellow colour. LOD of 18.3 ng/ml and 5.3 ng/ml were obtained for methyl-paraoxon and chlorpyrifos-oxon respectively.

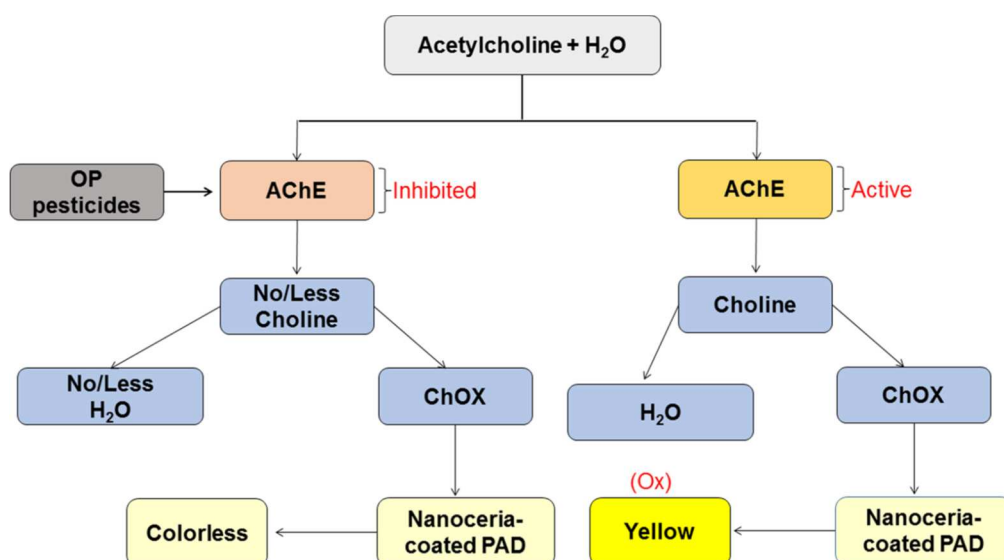


Figure 23. Principle of a paper-based enzymatic colorimetric biosensor for the analysis of organophosphate pesticides using nanoceria [112] (This article is licensed under a Creative Commons Attribution 4.0 International License).

To overcome some of the drawbacks of colorimetric biosensors, dual mode sensors gradually received extensive attention, especially dual colorimetric/fluorimetric sensors.

5. Dual enzymatic colorimetric/fluorimetric biosensors

The optical detection in dual enzymatic sensors is based on two modes of detection. Dual detection provides greater accuracy and reliability than single mode detection, because the two signals are independent of each other. One verifies and confirms the results of the other. Dual mode could eliminate matrix interference, reduce false positive and false negative results [113].

Colorimetric and fluorimetric is the most popular dual platform. Both signals could be readable by naked eyes, or by readers not so sophisticated. This dual mode combined the simplicity and rapidness of colorimetric methods and the sensitivity of fluorescent sensors.

Table 2 presents some examples of dual enzymatic colorimetric and fluorimetric sensors for the detection of pesticides. In most of the cases, nanomaterials were used as enhancers for the signal or even directly as transducers for dual colorimetric/fluorimetric biosensors.

Table 2. Different fluorimetric and colorimetric (dual) enzymatic sensors for the detection of pesticides.

Analyte	Enzyme	Construction of the sensor/Sensing principle	Matrices	LOD	References
Trichlorfos	AChE	Oxidase-like 2D fluorescence nanozyme (PtPdNPs@g-C ₃ N ₄) OPD: colorimetric substrate Smartphone	Tea, apple, orange, cabbage	Colorimetric: 0.083 ng/ml Fluorimetric: 0.033 ng/ml	[114]
Chlorpyrifos	ALP	p-AP + DAMO => Si BNPs => orange colour and fluorimetric signals Inhibitor: pale orange and weak fluorescence	Tap water, lake water, baby cabbage and apple	Colorimetric: 0.57 ng/ml Fluorimetric: 0.21 ng/ml	[115]
Paraoxon	AChE	DTNB: colorimetric substrate TNB => fluorescence of Ti ₃ C ₂ MQDs quenched	Soil	0.20 µg/L	[116]
Dichlorvos, trichlorfon, and paraoxon,	AChE	IDA => changes in fluorescence signal and aggregation of AuNPs => changing the color from red to blue	Pear, Chinese cabbage	0.0032 mg/kg, 0.0096 mg/kg, and 0.0074 mg/kg (fluorimetric), 0.0120 mg/kg, 0.0224 mg/kg, and 0.0106 mg/kg (colorimetric)	[117]
Parathion methyl	AChE	Quenching of GQDs fluorescence with AgNPs (absorbers). AgNPs aggregation: colour change	water, apple, and carrot samples	0.017 µg/L	[118]
Acephate	ALP	AuNCs-SiO ₂ : strong fluorescence and light brown colour => Quenched by MnO ₂ (MnO ₂ -AuNCs-SiO ₂ nanocomposites) and deeper brown	Tap water, lake water, baby cabbage and apple samples	0.4 µg/l in the fluorescence detection, 0.09 µg/l in colorimetric	[119]
Paraoxon	AChE	DTNB + TCh => yellow-coloured TNBA => TNBA quenched the fluorescence of CDs	Water, rice, and cabbage	0.4 ng/ml	[120]
Carbaryl	AChE	TCh transform RB-AgNPs (yellow) and unquenched the fluorescence of RB simultaneously.	Tomato, apple and river water	0.023 ng/L	[121]
Carbaryl, diazinon, malathion, and phorate	AChE	TCh => RB-AuNPs from red to blue and turn on fluorescence of RB.	Agricultural products and river water	0.1, 0.1, 0.3, and 1 µg/l	[122]

ALP: alkaline phosphatase; DAMO: N-[3-(trimethoxysilyl) propyl]ethylenediamine; DTNB: 5,5-dithiobis (2-nitrobenzoic acid); GQDs: graphene quantum dots; OPD: o-phenylenediamine; RB: rhodamine B; TNBA: 5-thio-2-nitrobenzoic acid; Si BNPs silicon based nanoparticles.

Li et al. developed a dual colorimetric/fluorimetric sensor for the detection of paraoxon based on acetylcholinesterase inhibition by pesticides [120]. The substrate DTNB was decomposed by TCh to form a yellow coloured product, 5-thio-2-nitrobenzoic acid (TNBA) (**Figure 24**). Thus TNBA quenched the fluorescence of carbon dots (CDs). In the presence of paraoxon, less TCh was produced and consequently less TNBA; the absorbance decreased and the fluorescence of CDs increased. The two signals fluorimetric and colorimetric moved in opposite directions as a function of paraoxon concentrations. **Figure 25a** showed the UV/visible absorption spectra in relation to the concentrations of paraoxon. The colorimetric signal could be read by naked eyes and therefore could be used as a first screening step for field applications (**Figure 25b**). Then the fluorescence signal could be used to confirm the first visual reading, with a greater sensitivity (**Figure 25c**). A good linear relationship could be observed between inhibition efficiency (IE) and the concentration of paraoxon (**Figure 25d**). The LOD was reported as 0.4 ng/ml. This biosensor was applied to actual samples, water, rice, and cabbage. The selectivity towards usual components of agricultural and biological samples (eg. Na⁺, K⁺, glucose, lactose, bovine serum albumine) was high.

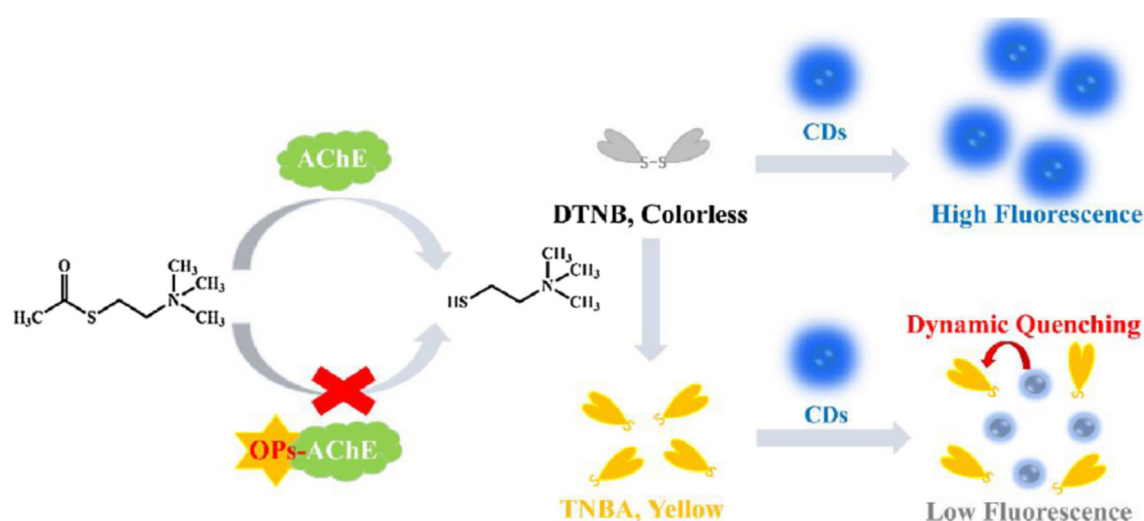
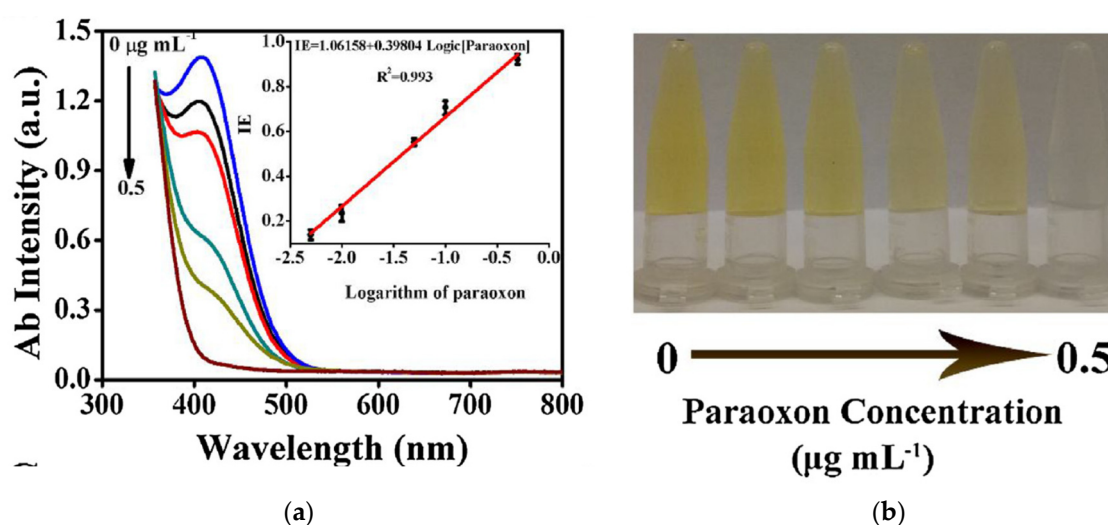


Figure 24. Schematic principle of the dual enzymatic colorimetric/fluorimetric sensor for the detection of organophosphate pesticides. Reprinted with permission from [120]. Copyright © 2018 Elsevier.



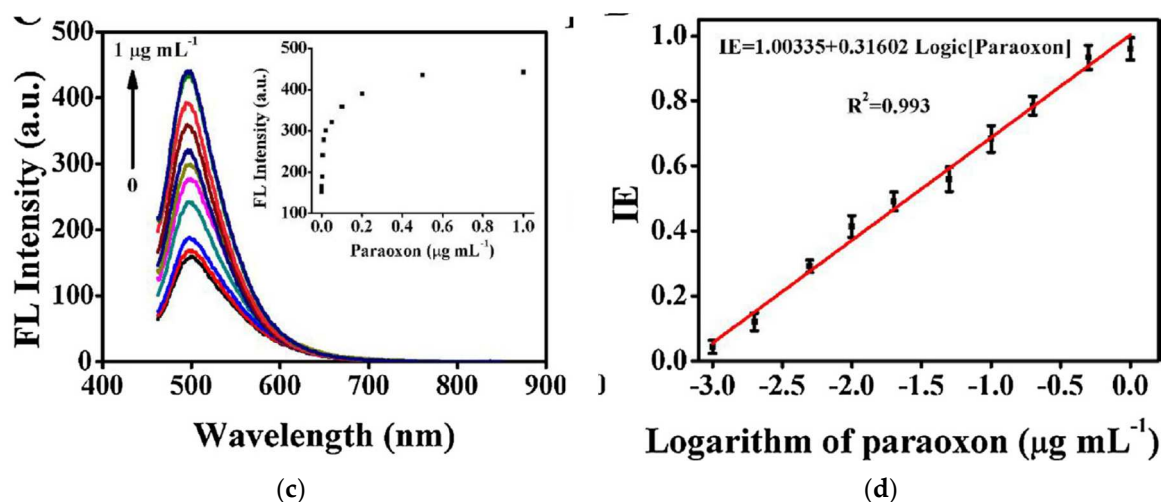


Figure 25. Dual enzymatic colorimetric/fluorimetric sensor for the detection of organophosphate pesticides. (a) UV-visible absorption spectra in relation to paraoxon concentrations from 0 to 0.5 µg/ml (b) Visual reading under daylight for the corresponding concentrations of paraoxon (c) Fluorescence spectra in relation to paraoxon concentrations from 0 to 1 µg/ml (d) Linear plot of inhibition efficiency (IE) in relation to logarithm of paraoxon concentrations. Reprinted with the permission from [120]. Copyright © 2018 Elsevier.

A dual colorimetric and fluorimetric enzymatic sensor was developed for the detection of methyl parathion (MP) [118]. The inner filtration effect (IFE) was observed when an absorber (ie. silver nanoparticles (AgNPs)) absorbed the fluorescence of the fluorophore (ie. graphene quantum dots (GQDs)). Furthermore the complex GQDs-AgNPs absorbed at 390 nm. Fluorescence decreased when the MP concentration increased (**Figure 26a,c**), while the absorbance increased when the MP concentration increased (**Figure 26b,d**). Indeed, TCh produced by AChE activity reacted with AgNPs which aggregated. Therefore the fluorescence of GQDs was recovered and the absorbance decreased. In the presence of MP, the AChE activity was inhibited. Therefore less TCh was produced, the fluorescence decreased and the aggregation of AgNPs led to an increase in the absorbance. The LOD of 0.017 µg/l reported for the fluorimetric assay was much better than the LOD of 0.2 µg/l for the colorimetric assay. The colorimetric assay is readable by naked eyes.

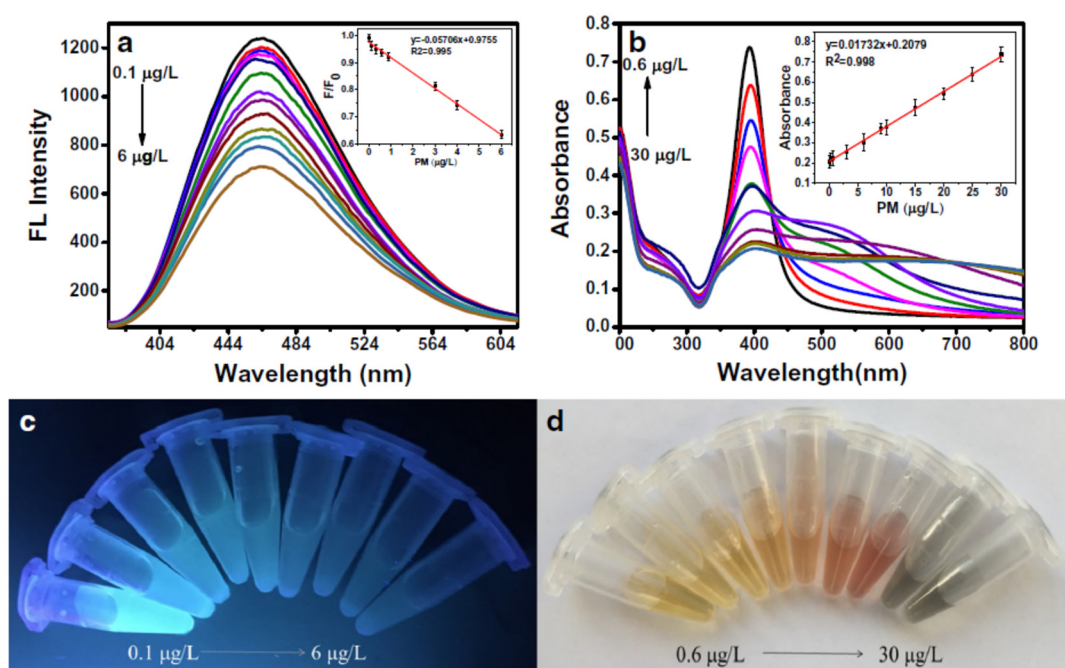


Figure 26. The effect of different concentrations of methyl paration on the fluorescence intensity (a, c) and the absorbance (b) and on the colorimetric detection of MP (d). Reprinted from permission from [118]. Copyright © 2020 Springer-Verlag GmbH Germany, part of Springer Nature.

Cai et al. developed a dual ALP sensor based on MnO_2 -AuNCs- SiO_2 nanocomposites for the detection of acephate (**Figure 27**) [119]. Pure gold nanoclusters (AuNCs) exhibited weak fluorescence, while strong fluorescence was emitted when AuNCs aggregated on the surface of silicon oxide nanoparticles (SiO_2 NPs). Due to IFE, manganese oxide (MnO_2) nanosheets quenched the fluorescence of AuNCs- SiO_2 NPs when MnO_2 -AuNCs- SiO_2 composites were formed. Ascorbic acid, produced by ALP activity, was able to decompose MnO_2 nanosheets. In this case, the fluorescence of AuNCs- SiO_2 NPs was restored and the brown colour faded. In the presence of pesticides, less ascorbic acid was produced, less MnO_2 nanosheets were decomposed; thus the fluorescence was quenched by MnO_2 NPs and the colour was brown. The absorbance increased proportionally to the pesticide concentration while the fluorescence decreased. The LOD for fluorescence detection was reported as $0.4 \mu\text{g/l}$ and $0.09 \mu\text{g/L}$ for colorimetric analysis. Other pesticides, dimethoate, parathion-methyl, profenofos and isofenphos-methyl, were detected on the dual-sensor, even it was with different sensitivities from acephate because of different inhibitory effects.

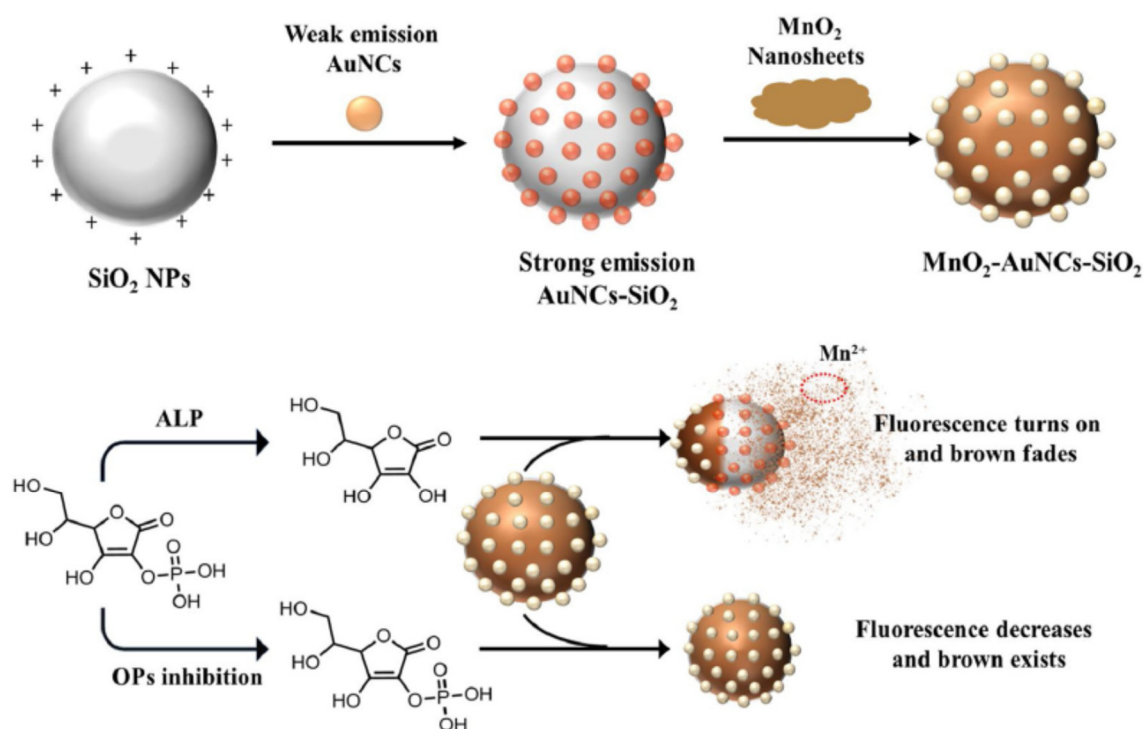


Figure 27. Schematic principle of the dual colorimetric/fluorimetric enzymatic sensor based on alkaline phosphatase and MnO_2 -AuNCs- SiO_2 composites. Reprinted with the permission from [119]. Copyright © 2020 Elsevier.

Jiang et al. developed a dual enzymatic sensor based on ALP inhibition for the detection of chlorpyrifos (**Figure 28**) [115]. p-aminophenol (p-AP) produced by ALP activity reacted with N-[3-(trimethoxysilyl) propyl]ethylenediamine (DAMO) to produce silicon based nanoparticles (Si BNPs). These Si BNPs could give colorimetric (orange color) and fluorimetric signals. In the presence of chlorpyrifos, the enzymatic activity is inhibited, thus less p-AP and Si BNPs were produced. The colorimetric (pale orange) and fluorimetric (weak fluorescence) signals decreased. Herein the two signals fluorimetric and colorimetric moved in the same direction as a function of chlorpyrifos concentrations. The LOD were reported as 0.70 ng/ml and 1.90 ng/ml for fluorimetric and colorimetric sensor respectively in lake water sample.

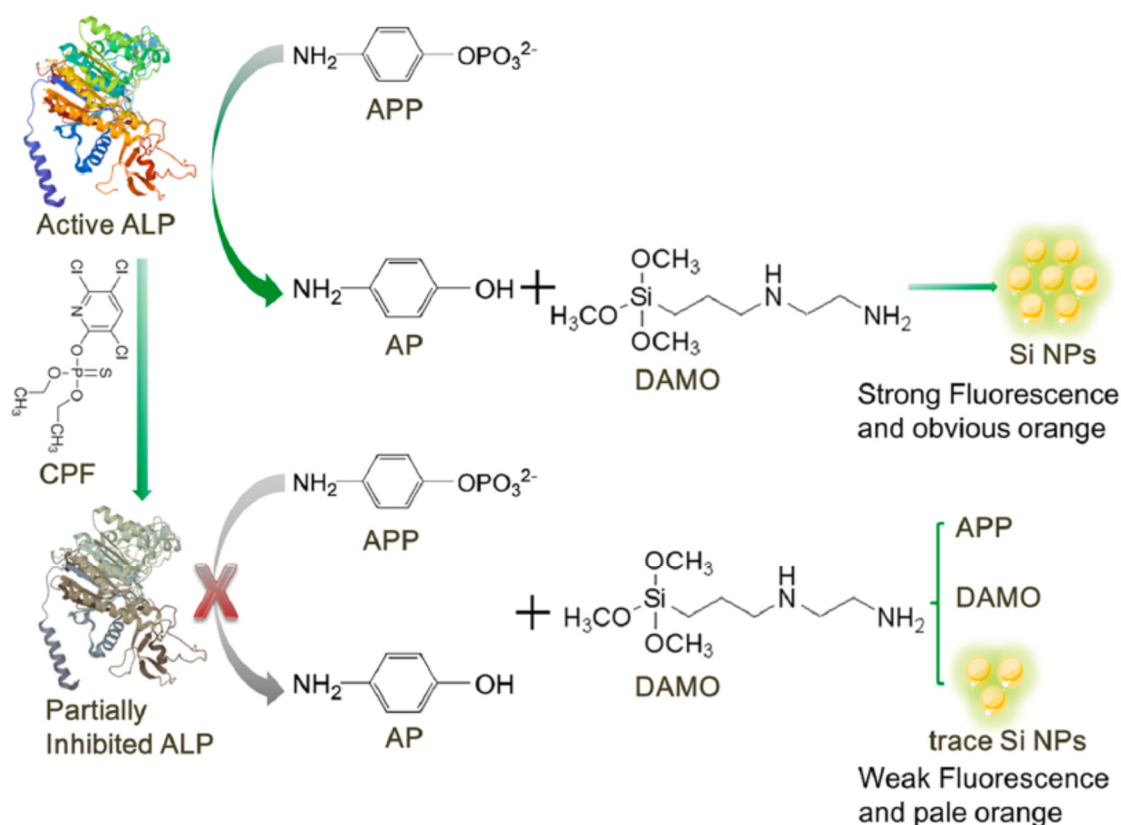


Figure 28. Schematic representation of dual enzymatic biosensor for the detection of chlorpyrifos. Reprinted with permission from [115]. Copyright © 2023 Elsevier.

A dual colorimetric and fluorimetric AChE based sensor was developed for the detection of OPPs in fruits and vegetables [114]. This sensor was based on the enzymatic activity of AChE, combined with an oxidase like activity of platinum palladium nanoparticles decorated graphite carbon nitride nanosheets (PtPdNPs@g-C₃N₄) (blue emission at 446 nm) (**Figure 29a**). PtPdNPs converted dissolved O₂ into O₂⁻ due to their oxidase like activity. Then the substrate (orthophenyldiamine (OPD)) which was not natively fluorescent, was oxidised by O₂⁻ into a product 2,3-diaminophenothiazine (DAP) which was fluorescent (yellow emission at 550 nm). When ATCh was transformed into TCh by the AChE activity, TCh and OPD competed to react with O₂⁻. In the presence of an OPP, the AChE activity is inhibited. Therefore less TCh was produced, so more O₂⁻ were available to react with OPD. Therefore more DAP was produced, which resulted in more strong fluorescence and increased absorbance (from light yellow to dark yellow). Again the two signals fluorimetric and colorimetric moved in the same direction as a function of OPP concentrations. The absorption and the fluorescence intensities were proportional to Trichlorfos (Trich) concentrations (**Figure 29b**). For field applications, a portable filter paper smartphone-based sensor was developed. Images of colorimetric and fluorimetric signals were taken and analysed with an application to convert images into RGB values. Very low LOD were reported for colorimetric test 0.083 ng/ml and fluorimetric test 0.033 ng/ml.

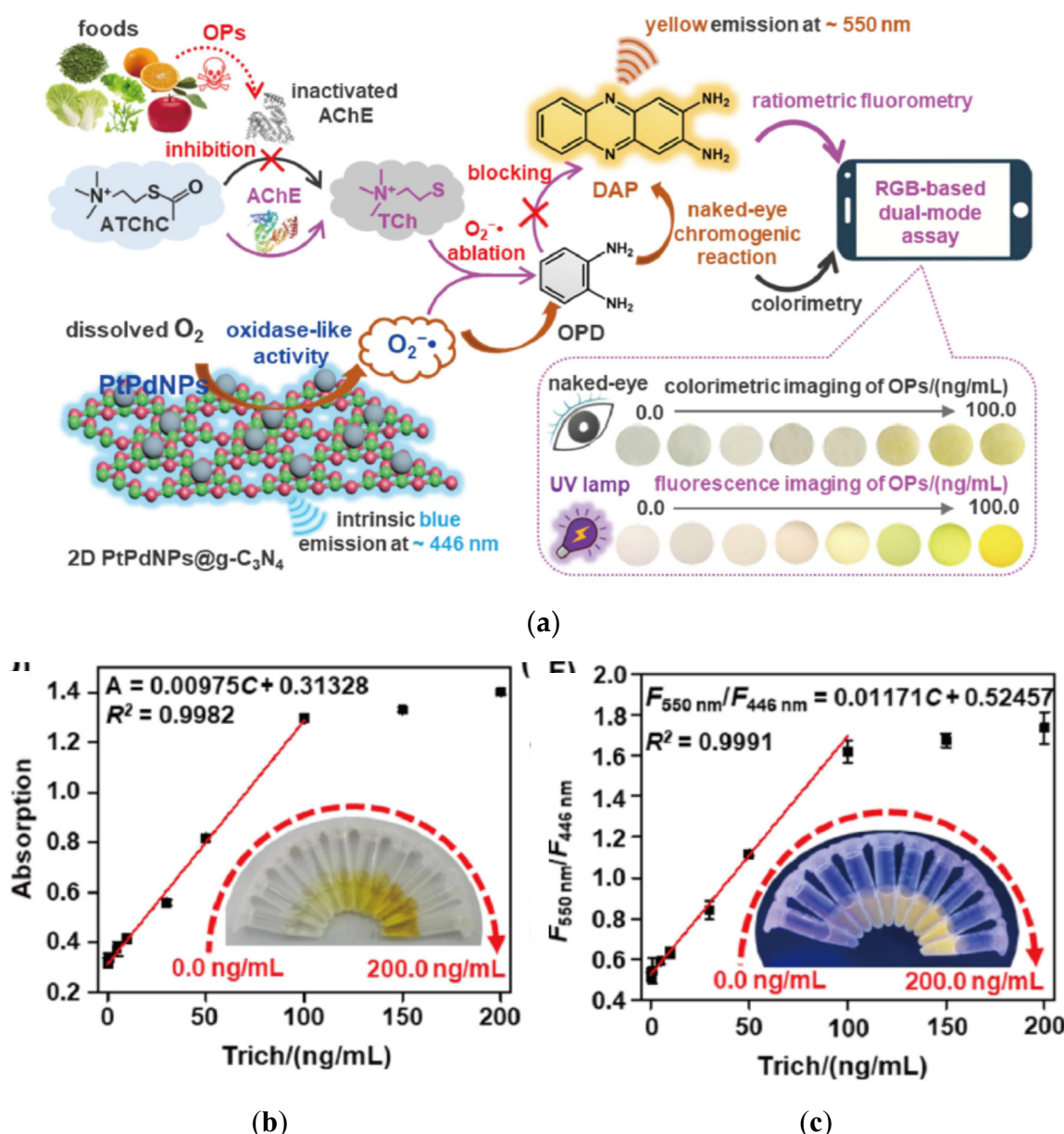


Figure 29. Dual assay for the detection of organophosphate pesticides (a) Schematic principle of the dual colorimetric and fluorimetric assay based on AChE inhibition by pesticides (b) Plot of the absorbance in relation to trichlorfos concentrations from 0 to 200 ng/ml (colorimetric test) (c) Plot of the fluorescence ratio $F_{550 \text{ nm}}/F_{446 \text{ nm}}$ in relation to trichlorfos concentrations from 0 to 200 ng/ml (fluorimetric test). Reprinted and modified with the permission from [114]. Copyright © 2023 Elsevier.

6. Conclusions and future prospects

In summary, biosensors appeared to be relevant solutions for developing field-applicable, simple, fast and sensitive self-monitoring tools. Colorimetric enzymatic sensors were widely developed during the past ten years for the detection of pesticides in different matrices. This study discussed the different sensing principles of enzymatic colorimetric sensors. The main sensing principle involved the inhibition of enzymatic activity by the target analyte. The enzyme acetylcholinesterase was the most common used enzyme for the detection of pesticides. This review emphasised the increasing contribution of nanomaterials as a replacement for conventional colorimetric substrates (DTNB, TMB, IDA, ...). Furthermore nanomaterials could be used to increase the signal that allowed to develop very sensitive methods. Gold nanoparticles represented the most

usual alternative to colorimetric traditional substrates. The limits of detection of gold nanoparticles based biosensors were very low, well below regulatory limits.

Dual-mode sensors have gradually received extensive attention and have been applied for the detection of pesticides. In this review, dual colorimetric/fluorimetric sensors for the detection of pesticides were presented. Dual detection provides greater accuracy and reliability than single mode detection, because the two signals are independent of each other. One verifies and confirms the results of the other. Dual mode could eliminate matrix interference, reduce false positive and false negative results.

The detection performance of colorimetric and dual colorimetric/fluorimetric sensors, and their applications in plant and animal derived food products, were discussed. The performance of colorimetric and dual enzymatic biosensors has improved considerably in recent years, thanks in particular to the development of nanomaterials and microfluidics.

However some challenges remain with enzymatic biosensors. A biosensor is constituted of two main elements, the bioreceptor and the transducer. Both of them participate to the performance of the developed biosensor. Acetylcholinesterase is not specific for pesticides; other chemical products (eg. neurotoxic agents (sarin)) could inhibit its activity and therefore be detected by AChE biosensors. The enzyme stability is also a critical point for the development of field sensors. More reliable immobilization protocols (polymers, nanomaterials) should be developed. The production of recombinant AChE could be a solution to develop more sensitive and selective biosensors for the detection of pesticides. Moreover, efficient immobilization method improves enzyme activity and stability, but also biosensor sensitivity. The enzyme activity is strongly correlated to the native structure of the enzyme that should not be degraded during immobilization.

Furthermore, the cost of nanomaterials could be very high. So even if nanomaterials could help to produce more sensitive biosensors, the cost of sensors for on site applications should be taken into account.

To reinforce the monitoring of pesticides on the field, the portability should be improved. It could be performed by the development of biosensors readable with smartphones. Indeed the colorimetric reading could be performed visually, with a classical spectrophotometer or more recently with a smartphone. Smartphones are emerging as potential replacements for the conventional laboratory instruments (ie. Spectrophotometer) [123]. Smartphones are widely used in everyday life, easy to use and portable. These features enable to develop biosensors suitable for field use. Free applications and software are available to convert images into quantifiable, usable data (ie. RGB principle). Disposable test strips (eg. Paper-based analytical devices) could be assessed by naked eyes, with a portable spectrophotometer or even better with smartphone. These biosensors are cost-effective, easy to use, and portable. Therefore lateral flow assays are promising tools for self-monitoring of pesticides in the industry.

The second perspective is to replace the enzyme in the biosensor by nanomaterials due to their enzyme-like properties (nanozymes). Nanozymes could be used to develop biosensors in combination with natural enzymes (tandem catalytic performance) like acetylcholinesterase [50, 77] or could replace the native enzyme [124]. Nanozymes became a promising alternative to natural enzymes in the past decade for the detection of pesticides, especially because of the better stability to environmental conditions [29].

Finally the literature on the development of biosensors and especially enzymatic biosensors for the detection of pesticides is extensive. However, these methods are not currently used for routine monitoring [125]. Many developed sensors remain at the step of proof of concept only. Sometimes the biosensor was not tested in actual conditions, with agricultural or biological samples. In some cases, one or two samples were tested to show the applicability. However during these research developments, no full validation were implemented. The limits of detection were determined mostly in buffer, which was a simpler matrix than biological or agricultural matrices. Therefore LOD were probably underestimated. Complex sample pre-treatment for some biological matrices could be an obstacle for the development of field biosensors.

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