History and New Developments of Assays for Cholinesterase Activity and Inhibition

ARTICLE *in* CHEMICAL REVIEWS · SEPTEMBER 2010

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History and New Developments of Assays for Cholinesterase Activity and Inhibition

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Received June 7, 2009

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1. Introduction

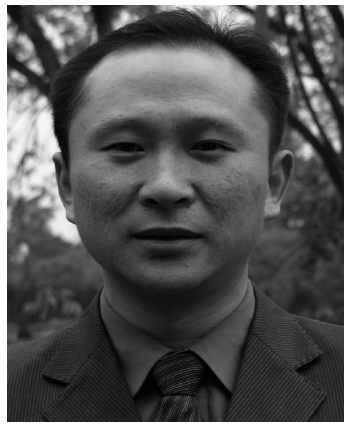
The cholinesterases (ChE's) are key enzymes in a range of important areas such as neurobiology, toxicology and pharmacology. Of these, two major groups, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), play important roles in human and animal function and health. AChE, also known as true ChE, is present mainly in the central nervous system.[1] It is bound to the cellular membranes of excitable tissues and is involved in nerve transmission processes. Its chief biological function is to catalyze the hydrolysis of the neurotransmitter acetylcholine into choline, which is a reaction necessary to allow a cholinergic neuron to return to its resting state after activation. AChE is found in the membranes of red blood cells, where it is known as erythrocyte ChE. BChE, also known as pseudocholinesterase, plasma or serum ChE, is primarily found in plasma, liver, and muscle tissues. Its biological function is also uncertain. Although this enzyme has a similar molecular structure to AChE, it is characterized by a different, but overlapping, spectrum of substrate specificities in which AChE preferentially hydrolyzes acetyl esters such as acetylcholine, whereas BChE hydrolyzes butyrylcholine:[2−4]

$$\underset{\text{acetylcholine}}{(CH_3)_3N^+CH_2CH_2O{-}CO{-}CH_3} + H_2O \xrightarrow{\text{AChE}} \underset{\text{choline}}{(CH_3)_3N^+CH_2CH_2OH} + \underset{\text{acetic acid}}{CH_3COOH}$$

$$\underset{\text{butyrylcholine}}{(CH_3)_3N^+CH_2CH_2O{-}CO{-}(CH_2)_2CH_3} + H_2O \xrightarrow{\text{BChE}} \underset{\text{choline}}{(CH_3)_3N^+CH_2CH_2OH} + \underset{\text{butyric acid}}{CH_3(CH_2)_2COOH}$$

Assays for ChE activity and its inhibition are applicable to a very broad range of fields, including the pharmaceutical, healthcare, food, and agricultural industries, environmental monitoring, and quality control. Both qualitative and quantitative assays of ChE activity or inhibition have been reviewed extensively.[5−16] These assays can either be observed visually or measured by a variety of optical and electrochemical techniques or by piezoelectric devices. However, most of these reviews focused on biosensor areas. There is a great gap between traditional assays and newly developed

10.1021/cr900214c
Published on Web 07/01/2010



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Nongyue He obtained both his B.S. and M.Sc. degrees from Hunan Normal University and his Ph.D. degree from Nanjing University, China. Then he entered Southeast University as a postdoctoral researcher and has been a full professor in the department of Biomedical Science and Medical Engineering School, of the Southeast University, since 2000. His research interests are focused on developing biochips and biosensors, functional nanomaterials, controlled drug release systems, and tissue engineering applications. Currently, a particular focus of his research is to the development of high-throughput bioassay methods and related devices or instruments. He gained the Award for Excellent Teachers in Chinese Universities (Ministry of Education of China); the Award for Advance in Science and Technology of Hunan Province Government, China; Chien-Shiung Wu Award granted by Southeast University; and the First Award of Endoscopic Medical Tech-Science in China. He has published about 150 papers and 6 books and has over 30 patents.

techniques such as biosensors. It is well-known that no one technique is suitable for all applications. The ultimate purpose of the application is important for ensuring a judicious choice assay. This decision is governed by several factors including the choice of enzyme resources, immobilization methods, enzyme substrates, transducers, buffer, and pH, most of which are often neglected.

The purpose of this review is to encompass as many as possible of these relevant aspects, albeit within certain limits. It is useful to focus on topics that bridge the gap between

Jun-Jie Zhu is Professor of Chemistry at Nanjing University, China. He was born in Shanghai, China, in 1960 and received his B.S. (1984) and Ph.D. (1993) degrees from the Department of Chemistry, Nanjing University, under the supervision of Professor Hong Gao. In 1993, he began his academic career at Nanjing University. Between 1998 and 1999, he was a postdoctoral fellow with Professor Aharon Gedanken in Bar-Ilan University, Israel. His current research interests are in the fields of materials and biological analytical chemistry, mainly focused on the study of biological analytical chemistry including bioelectrochemistry, nanoelectrochemistry, and the fabrication of biosensors and nanosensors. The syntheses of nanoparticles using sonochemistry, sonoelectrochemistry, and microwave chemistry method are also currently being investigated.

traditional assays and newly developed techniques, and hence, assays concerning ChE activity and inhibition are reviewed with particular emphasis on AChE and BChE. The more general assay approaches are introduced, and research on ChE-based biosensors for the determination of organophosphorous (OP), organochlorine pesticides, or other toxic compounds for food and environmental applications is highlighted. New material science and biomimetic techniques have contributed greatly to the development of assays for catalytic activity as well as the inhibition of ChE. Organic-phase analysis is also discussed. The review finishes with a short discussion of the outlook and future perspectives in both research and development. With respect to the history and development of ChE-related assays, one of the earliest literature reports has been tracked back as far as 1949. Although most of of the relevant literature concerning traditional ChE assays appeared in the last 40 years of the 20th century, many new and important developments occurred during the past 20 years.

2. ChE Enzymes: A General Background

2.1. ChE Enzymes

Although the assessment of activity and inhibition of ChEs has attracted a great deal of attention, most of these studies used unpurified enzymes. The danger is that these could give misleading results due to the crude extracts probably being contaminated with carboxylesterases or other esterases.[16] Commercially available ChE enzymes from *Drosophila melanogaster* and *Electric eel* are the most widely used for bioassay applications because of the high inhibition sensitivity that OP compounds and carbamate pesticides have on them. In addition, genetically engineered ChEs have also been chosen to develop biosensor applications because of their high sensitivity toward insecticides.[17,18] However, it should be noted that, because ChEs are inherently unstable due to being rendered inactive once isolated from their native

biological environment, the range of their real applications is severely hindered. Temperature, detergents, and pH are among the parameters that further influence enzyme stability. On the basis of existing theoretical models, the stabilization of proteins against unfolding can be achieved by physical confinement inside relatively small cages.[19] The unfolded configurations are thermodynamically disfavored in such confined spaces. In the study by Sotiropoulou et al., the use of two different meso/nanomaterials for the immobilization of m-AChE shows that there is a decrease in the leaching of the protein from the biosensor membrane into the solution as well as a drastic increase in the operational stability of the resulting biosensor.[20]

Certain plant esterases, including that from wheat germ, hydrolyze glyceryl, aromatic, and choline esters. Since acetates are cleaved more rapidly than esters of the higher acid homologues, they are called acetyl enzymes. Plant acetylesterases were also found to be inhibited by OP compounds.[21,22]

2.2. Various Substrates for ChE Assays

Apart from natural substrates acetylcholine, ChE also hydrolyzes many other esters, both natural and synthesized, including esters of thiocholine such as acetylthiocholine (ATCh), butyrylthiocholine (BTCh), propionylthiocholine, acetyl-β-methylthiocholine, *o*-nitrophenylacetate, indophenylacetate, *p*-aminophenyl acetate, and α-naphthyl acetate. Many of these have been explored with respect to applying different ChE assay strategies.

As mentioned in the introduction, AChE and BChE differ in their substrate specificity. AChE hydrolyzes acetylcholine at the highest rate, hydrolyzes propionylcholine and acetyl-3-methylcholine somewhat more slowly, and has almost no influence on the rate of hydrolysis of butyrylcholine and benzoylcholine.[23,24] BChE hydrolyzes butyrylcholine at the highest rate; hydrolyzes propionylcholine, acetylcholine, benzoylcholine, succinylcholine, mivacurium, and ester-type local anesthetics such as procaine, chloroprocaine, tetracaine, cocaine, and heroin somewhat more slowly; and has no influence on the rate of hydrolysis of acetyl-3-methylcholine.[25]

Of the several thiocholine alkanoyl esters that were synthesized and explored as substrates for the ChE human serum assay using the Ellman reaction,[26] isobutyryl and cyclohexane−carboxyl esters showed the best efficiency. For isobutyryl and cyclohexane−carboxyl esters, spontaneous hydrolysis in the aqueous phase was ∼1/25 and ∼1/175 slower, respectively, than enzymatic hydrolysis. Their K_m values were almost equivalent. The substrates were stable in aqueous solution and in the solid state as the iodides for at least 5 years at 5 °C.

The substrate specificity of brain AChE in adult *Carassius auratus* fish and its sensitivity to carbamate insecticides were investigated in vitro by Liu et al.[27] Four substrates were hydrolyzed by AChE giving the following order: ATCh iodide > β-methylthiocholine iodide > propionythiocholine iodide > BTCh iodide. The maximum velocity of AChE hydrolyzing ATCh iodide was the highest among the four substrates.

Four commonly used substrates (benzoylcholine, BTCh, acetylcholine, and propionylthiocholine) were used to assay a large number of patient samples in order to assess their ability to identify known buccinylcholine-sensitive patients.[28] The method is based upon that developed by Dietz et al.[29] Propionylthiocholine proved the best substrate for this purpose. It was capable of identifying >90% of affected individuals with no false positives. Acetylcholine and BTCh were slightly less effective substrates, with benzoylcholine giving little useful information.

2.3. Reactivation of ChE Activity

Biosensors based on ChE inhibition have been developed for monitoring pesticides in food and water samples. However, the strong inhibition of the enzyme makes them usable as biosensors in a single-serve practical application only. An attempt has been made to overcome this drawback by reactivation of the enzyme for repeated use.

Pyridine 2-aldoxime methiodide (2-PAM) has been the substrate of choice for this purpose.[30,31] However, the efficiency of the reactivation proved inadequate. The following two oximes 1,1 V-trimethylene bis-4-formylpyridinium bromide dioxime (TMB-4) and 2-PAM were compared for the reactivation of immobilized AChE.[32] TMB-4 was found to be more efficient under repeated use; it retained >60% of initial activity after 11 reuses. On the other hand, with 2-PAM, the activity retention dropped to <50% after only 6 reuses. Reactivation must be effected within 10 min after each analysis, which reduces the efficiency of reactivation further. Reactivation of phosphorylated AChE using an oxime can be represented by the following equation:

$$\underset{\text{phosphorylated enzyme}}{\text{enzyme}-\text{O}-\text{P}(=\text{O})(\text{R}_1)(-\text{OR}_2)} + \underset{\text{oxime}}{\text{O}-\text{N}=\text{CHR}} \rightarrow \underset{\text{phosphorylated oxime}}{\text{CHR}=\text{N}-\text{O}-\text{P}(=\text{O})(\text{R}_1)(-\text{OR}_2)} + \underset{\text{reactivated enzyme}}{\text{enzyme}}$$

An improvement of the AChE-based assay reactivation using selective reactivation of the AChE activity after previous inhibition has been described.[33] Four OPs, paraoxon−ethyl, paraoxon−methyl, trichlorfon, and methamidophos, as well as the three most easily available reactivators, HI-6, obidoxime, pralidoxime, were used. Subsequently, the most significant identifications were based on methamidophos-inhibited AChE reactivation using HI-6 or pralidoxime and paraoxon−ethyl inhibited AChE by obidoxime.

Indeed reactivator performance is a great challenge for ChE-based assays. Although regeneration is always partial, some authors refer to "quasi-total" or "total" regeneration.[34] The reactivation step requires time, ranging from 1 h to overnight depending on the degree of inhibition. The issues of poor reproducibility and cost have to be solved before this technique can be applied to the development of applicable assays.

2.4. Inhibition of ChE Activity and Cessation of Enzymatic Reactivity

AChE hydrolyzes acetylcholine at a higher rate than butyrylcholine but is inhibited by excess substrate. On the other hand, although BChE hydrolyzes butyrylcholine at a higher rate than acetylcholine, it is not inhibited by excess substrate. Both of them are inhibited by eserine, choline, quinidine, tetramethyl ammonium ions, acetylcholine, *p*-carboxyphenyltrimethylammonium iodide, trimethyl (*p*-aminophenyl) ammonium chloride hydrochloride, neostigmine, ethionamide, dimethoate, phosphatidylserine, prostigmine, ammonium salts, and various organophosphorus, organochlorine, and carbamate pesticides.

A ChE inhibitor (or anticholinesterase) is known to suppress the activity of the enzyme. AChE inhibitors are traditionally divided into two groups: reversible and irreversible inhibitors. Reversible inhibitors form noncovalent bonds only, and almost all contain at least one positively charged nitrogen group. Therefore, they inhibit ChE, at least in part, through occupation of the anionic site.[35] Carbamates and OPs are two major classes of irreversible inhibitors that either carbamylate or phosphorylate the serine residue of the active site using both covalent and noncovalent bonds. These inhibitors have been used as agricultural pesticides and chemical warfare agents. They can also be used in the treatment of myasthenia gravis, glaucoma, and Alzheimer's disease. Inhibitors such as benzethonium chloride, benzalkonium bromide, quinidine, and eserine often are used to terminate the enzymatic reaction when ChE activity is assayed.

It should be noted furthermore that AChE and BChE differ in their sensitivity to various inhibitors. The distinction between them can be made by using inhibitors that selectively block their activity.[36] For example, when AChE activity is measured in diluted whole blood, a selective BChE inhibitor such as a phenothiazine derivative, e.g., ethopropazine, is often used.

3. Assay of ChE Activity and Inhibition

Numerous methods have been reported for the determination of both ChE activity and inhibition. Most of these either measure the change in pH or utilize colorimetric, spectrophotometric, fluorometric, radiometric, or electrochemical techniques. New material-based techniques and nanotechnology have made a great contribution to the development of various assays for ChE activity and inhibition.

3.1. Spectrometric Assays

Although there are many types of ChE assays, the most prevalent type of assay in use today is still the spectrometric-based assay.

3.1.1. UV−Vis Assays

In the early development stage of spectrophotometric assays, a "remaining substrate" method based on the colorimetric determination of iron(III) hydroxamate was described.[37−39] Hydroxylamine reacts with the remaining acetylcholine and is converted to acetylhydroxamic acid. The latter forms a red complex with Fe^{3+} that can be photometrically monitored in order to assay ChE activity. This technique suffers from the disadvantage of needing large reaction volumes and the fact that it does not measure the rate of disappearance of the substrate.

Undoubtedly, the most widely used ChE assay is the Ellman method.[40] This assay uses the thiol esters ATCh or BTCh as well as a synthetic substrate instead of the oxy ester acetylcholine or butyrylcholine. AChE or BChE hydrolyzes the ATCh or BTCh to produce thiocholine, which in turn reacts with dithiobisnitrobenzoate (DTNB) to produce a yellow 5-thio-2-nitrobenzoic acid (TNB) (Scheme 1). The color intensity of the product is measured at 412 nm, and it is proportional to the enzyme activity. These coupled reactions are represented by the following equations:

$$(CH_3)_3N^+CH_2CH_2S{-}CO{-}CH_3 + H_2O \xrightarrow{ChE} (CH_3)_3N^+{-}CH_2CH_2{-}SH + CH_3COOH$$

(ATCh; thiocholine; acetic acid)

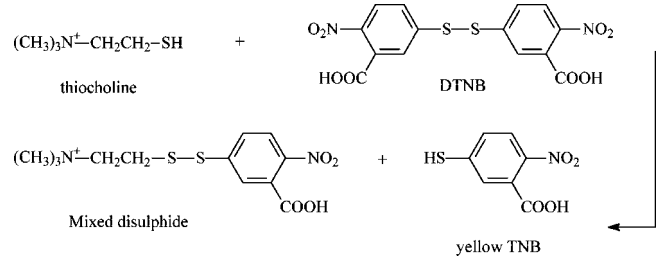
The main advantages of the Ellman method are simplicity, accuracy, a continuous increase in color density as a function of incubation time, and relatively low cost.[35] It is easily adaptable for automated analyzers or plate readers for the rapid processing of large numbers of samples. The Ellman method can also be adapted to measure ChE in the field by using a battery-powered colorimeter with a silicon carbide (blue) light-emitting-diode source.[41] The disadvantages include the facts that the −SH groups in the sample may react with DTNB and ATCh and the natural substrates are not identical from a kinetic point of view.

Several modifications for improving the performance of the Ellman method have been reported.[42,43] It was discovered that the optimal pH for ChE performance was pH 7.8−8.0.[43] However, the ATCh substrate was found to undergo spontaneous hydrolysis, which is accelerated by high temperature, alkaline pH, and high substrate concentration. Spontaneous ATCh hydrolysis increased by 2- and 4-fold as the reaction temperature was increased from 25 to 30 to 35 °C, respectively. For each degree increase in reaction temperature, enzyme activity increased ∼4%. As a compromise, an assay temperature of 30 °C was suggested. In addition, the DTNB is more stable at pH 7 than at pH 8, and thus, the recommendation is that the stock solution of DTNB be prepared at pH 7.[43]

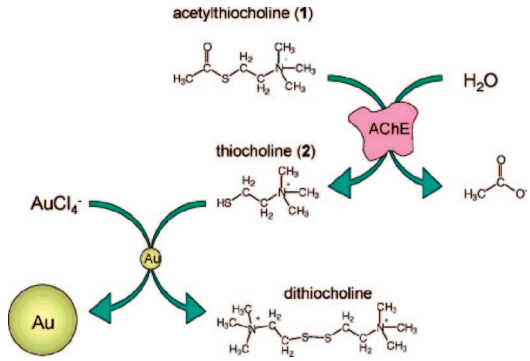
One drawback of the Ellman method is that the massive Soret absorbance of hemoglobin interferes with the absorbance of the reaction product TNB at 410 nm. Micellar chromophore interactions were utilized to overcome this. The TNB absorbance peak is shifted from 410 to 435 nm in the presence of the cationic detergent benzethonium chloride instead of using quinidine sulfate to stop enzyme action.[42] An added advantage is that the Soret band of hemoglobin is shifted to 405 nm and its peak intensity is decreased by about one-half due to the formation of methemoglobin. This results in an assay twice as precise, making it suitable for use in a routine laboratory with even a moderate-quality spectrophotometer.

An alternative method using dithiodinicotnic acid (DTNA) as the color reagent (absorbance at 344 nm) instead of DTNB was suggested.[44] Additionally, 2,2′-dithiodipyridine (2-PDS) has been reported as an alternative chromophore to DTNB for whole blood ChE assays because the product of the reaction, 2-thiopyridone, is measured at 343 nm, which mitigates hemoglobin interference.[10,45−47] However, a negative conclusion about 2-PDS has also been reported.[48] The authors found that DTNB did not influence serum ChE activity, whereas 2,2′-dithiodipyridine had an inhibitory

Scheme 1. Principle of the Ellman Method



Scheme 2. Detection of AChE Activity by Growing Au Nanoparticles[52]



effect. The lowering of the molar extinction coefficients observed in the presence of physostignine may be due to the reaction between thiolate ions with the carbamate moieties. The use of DTNB is still recommended in this report,[48] especially where the quantitative aspects are significant.

A multienzyme procedure reported a procedure to overcome the spectral interference arising from the superposition of the Soret hemoglobin band where the AChE hydrolyzes acetylcholine. The resulting choline is measured using choline oxidase (ChO) coupled to peroxidase, with phenol and aminoantipyreneto to give a pink product with a maximum absorbance at 500 nm.[49−51] The report also confirmed that normal erythrocyte components such as catalase do not interfere.

The use of metal and semiconductor nanoparticles for the optical and electrical sensing of biorecognition processes is developing rapidly.[52] Pavlov et al. reported the AChE-stimulated growth of Au nanoparticles. AChE hydrolyzes ATCh to yield a reducing agent thiocholine that stimulates the catalytic enlargement of the Au nanoparticle seeds in the presence of $AuCl_4^-$ (Scheme 2, Figure 1). The reductive enlargement of the Au nanoparticles is dependent on both substrate concentration and enzyme activity. The catalytic growth of the Au nanoparticles is inhibited by 1,5-bis(4-allyldimethylammoniumphenyl)pentane-3-one dibromide or by paraoxon. This enables a colorimetric test for the AChE inhibitors. As a result, the system provides a novel nanotechnology-based sensing method for nerve gases.

Changes, due to aggregation in the proximity of colloidal gold, causes a large change in the absorption spectrum of

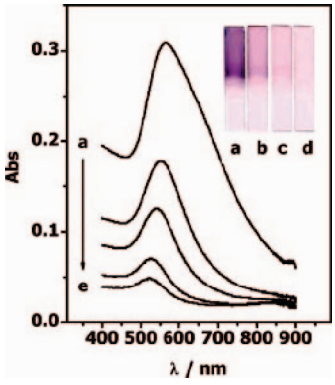
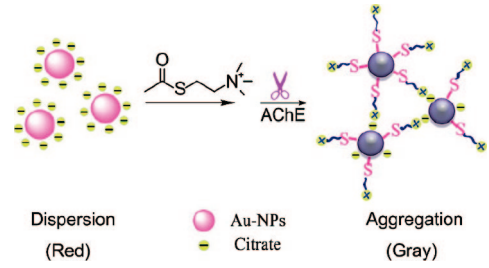


Figure 1. Absorption spectra and a photo corresponding to Au nanoparticle growth inhibition on glass supports in the presence of AChE, $HAuCl_4$, acetylthiocholine, and varying concentrations of 1,5-bis(4-allyldimethylammoniumphenyl)pentane-3-one dibromide. Reprinted with permission from ref 52. Copyright 2005 American Chemistry Society.

Scheme 3. Illustration of Colorimetric AChE Assay by Using Cross-Linking/Aggregation of Gold Nanoparticles Based on AChE-Catalyzed Hydrolysis of Acetylthiocholine (Reprinted with Permission from Ref 53; Copyright 2009 American Chemistry Society)



the colloidal suspension due to long-range coupling of surface plasmons. Wang et al. reported another interesting colorimetric assay where AChE catalyzes the hydrolysis of ATCh to give thiocholine, which induces the cross-linking/aggregation of gold nanoparticles. This in turn results in a red-shift of the plasmon absorption due to interparticle plasmon interactions (Scheme 3, Figure 2).[53] Using this method, not only can the activity of AChE be assayed but it also is useful for screening AChE inhibitors.

3.1.2. Fluorometric Assays

Fluorometric assays provide higher sensitivities and lower detection limits. Generally these are several orders of magnitude more sensitive than the corresponding chromogenic ones. Fluorescence chemicals are stable with a high degree of long-lived fluorescence intensity. They also have broad excitation wavelengths. Two fluorogenic substrates are compared for ChE assay: resorufin butyrate and indoxyl acetate,[54] both of which are nonfluorescent compounds that are hydrolyzed by ChE to highly fluorescent materials (Scheme 4). The rate of hydrolysis of the indoxyl acetate is much faster than that of resorufin esters. The added advantages of indoxyl acetate over resorufin acetate or butyrate are its greater stability toward spontaneous hydrolysis and the greater difference between the excitation and emission wavelengths. An advantage of the resorufin esters is the greater florescence of resorufin. This permits the assaying of lower substrate concentrations. In addition to ChE, acylase, acid phosphatase, and chymotrypsin all hydrolyzed the substrates to varying degrees. *N*-Methyl indoxyl acetate was also reported as being used to develop a fluorometric method for the determination of OP and chlorinated pesticides.[55] *N*-Methyl indoxyl acetate is cleaved by ChE to yield the highly fluorescent *N*-methyl indoxyl. The action of AChE

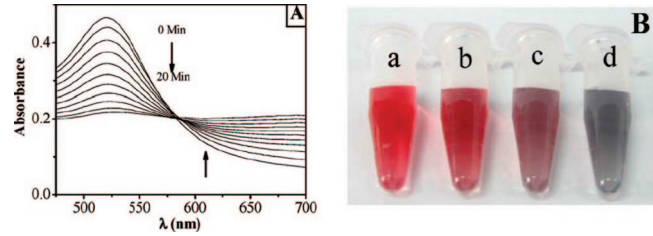
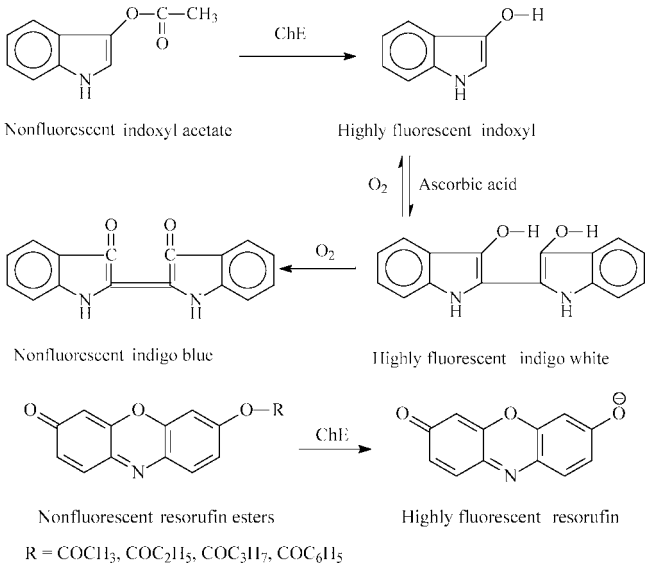


Figure 2. Colorimetric assay for AChE using Au nanoparticles. (A) Absorption spectra of Au nanoparticle solutions containing ATCh and those after hydrolyzing in the presence of AChE for different times. (B) Color changes for Au nanoparticle solutions containing ATCh with and without different amounts of AChE after incubation. Reprinted with permission from ref 53. Copyright 2009 American Chemistry Society.

Scheme 4. Fluorogenic Substrates for ChE Assay (Reprinted with Permission from Ref 54; Copyright 1965 American Chemistry Society)



and ChE on the indoxyl acetate and 2-naphthyl acetate was studied kinetically.[56] It's found that both enzymes convert the substrates to highly fluorescent products (3-hydroxyindole and 2-naphthol, respectively).

Parvari et al. developed a highly sensitive microfluorometric assay for ChE.[57] The thiocholine produced by the enzymatic hydrolysis of ATCh is reacted with the fluorogenic compound *N*-[4-(7-diethylamino-4-methylcoumarin-3-yl)phenyl]maleimide to yield an intensely blue fluorescent product with a fluorescence emission at 473 nm. The background emission caused by nonenzymatic hydrolysis of the substrate is relatively low. It does not exceed background values encountered in other methods. The advantages of this assay are the wide linear range of accuracy, the sensitivity (l00-fold higher than that of radiometric methods), and the low cost.

The Amplex Red Acetylcholine/AChE Assay Kit (Molecular Probes, Inc.) provides an ultrasensitive method for continuously monitoring AChE activity using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), a sensitive fluorogenic probe for H_2O_2. In the first step, AChE converts the acetylcholine to choline, and this is followed by choline being oxidized by ChO to betaine and H_2O_2. In the presence of horseradish peroxidase, the latter reacts with the Amplex Red reagent to generate the highly fluorescent product resorufin. There is little interference from autofluorescence in most biological samples since resorufin has absorption and fluorescence emission maxima of approximately 571 and 585 nm, respectively.

Compared with dye molecules, semiconductor nanoparticles, also called quantum dots (QDs), have unique fluorescence properties. Their size-controlled fluorescence properties and high fluorescence quantum yields makes them superior optical labels for biosensing. Controlling the photophysical properties of QDs by H_2O_2 may provide a new and versatile method for developing QD-based bioanalysis (Figure 3).[58] AChE hydrolyzes acetylcholine to choline, whereas ChO oxidizes choline to produce betaine and H_2O_2. The latter quenches the QD luminescence. Increasing the inhibitor concentration lowers the content of enzyme-generated H_2O_2 and, therefore, decreases the QD quenching rates.

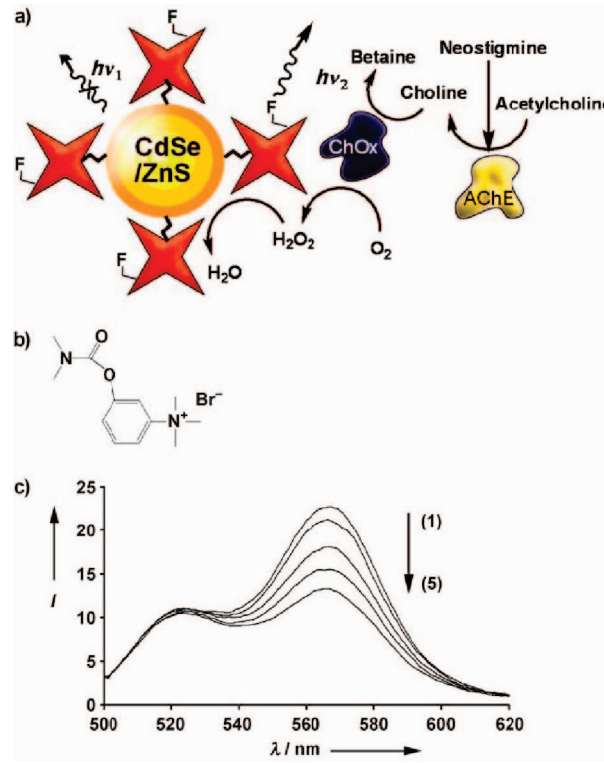


Figure 3. (a) Ratiometric analysis of AChE activity using the fluorophore-modified avidin-capped CdSe/ZnS QDs, and its inhibition by neostigmine. (b) Structure of neostigmine. (c) Time-dependent fluorescence changes upon interaction with ATCh, ChO, and acetylcholine. Reproduced with permission from ref 58. Copyright Wiley-VCH Verlag GmbH & Co. KGaA.

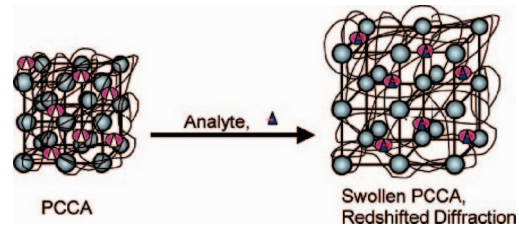


Figure 4. PCCA backbone is functionalized with a molecular recognition agent, which binds the analyte, actuating either shrinkage or swelling of the hydrogel network. Reprinted with permission from ref 59. Copyright 2005 American Chemistry Society.

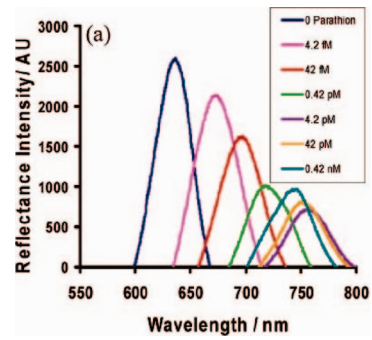


Figure 5. Diffraction response of AChE−PCCA to parathion solutions of varying concentrations. Reprinted with permission from ref 59. Copyright 2005 American Chemistry Society.

3.1.3. Diffractometric Assays

Walker and Asher developed a polymerized crystalline colloidal array (PCCA) photonic crystal-sensing material that is capable of sensing ultratrace concentrations of OPs (Figures 4 and 5).[59] A periodic array of colloidal particles is

embedded in a hydrogel network with a lattice spacing such that it diffracts visible light according to Bragg's equation. AChE is phosphorylated in the presence of parathion. This charged species creates a Donnan potential and swells the hydrogel network. The volume change in the hydrogel increases the lattice spacing of the embedded particle array and causes a red-shift in the wavelength of the diffracted light. The magnitude of the diffraction red-shift is proportional to the parathion concentration. This can then be monitored using a fiber-optic diode spectrometer with a reflectance probe.

3.1.4. Mass Spectrometric Assays

A paper describing a high-performance liquid chromatography−mass spectrometry (HPLC-MS) methodology for the screening of AChE inhibitors from natural extracts has also appeared.[60] The AChE activity of the sample is monitored using a postcolumn biochemical assay that is based on the separate, sequential mixing of AChE and acetylcholine, respectively, with the HPLC eluate. AChE inhibitors are detected by measuring a decrease in product formation using electrospray MS. The assay has shown it can identify an AChE inhibitor present in a crude extract of *Narcissus* c.v. "Bridal Crown". A sensitive and specific high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) assay for the determination of rivastigmine and its major metabolite NAP 226-90 has also been presented.[61] Detection was performed by using a turbo ion spray interface and positive ion multiple reaction monitoring in tandem mass spectrometry. Validation results demonstrate that rivastigmine and its metabolite concentrations can be accurately and precisely quantified in human EDTA plasma. This assay is now used to support clinical pharmacologic studies involving rivastigmine. Another report regarding this approach has also been published.[62]

3.2. Histochemical Localization of AChE

Since the introduction of the thiocholine method for the histochemical localization of AChE or BChE by Koelle and Friedenwald,[63] many modifications have been developed to improve it for either light or electron microscopy.[64] They included the addition of high concentrations of sodium sulfate and various selective inhibitors, preliminary fixation with formalin and incubation at low pH, omission of the final conversion of copper thiocholine sulfate to copper sulfide, and substitution of lead or gold for copper as the capturing agent. The most widely accepted innovation is that developed by Karnovsky and Roots.[65] Here, the enzymatically released thiocholine reduces ferricyanide to ferrocyanide, and this in turn is precipitated by cupric ion and citrate as cupric ferrocyanide, $Cu^{2+}Fe^{2+}(CN)_6$, Hatchett's brown. This modification has the advantage of directly producing a granular, readily visible precipitate. Their method was further improved by Erankö et al., who used lead complexed with trisacetate buffer as the trapping agent for the ferrocyanide ion, which is formed by the reduction of ferricyanide by the released thiocholine.[64] The resulting colloidal, faintly yellowish-white precipitate, $Pb_2Fe(CN)_6$, can be viewed directly by light or phase-contrast microscopy. Extremely precisely localized AChE was observed in cat superior cervical and stellate ganghia and at the motor end plates of mouse intercostal muscle.

Although Karnovsky and Roots' thiocholine−ferricyanide method is widely used for the localization of AChE, thiocholine reduces the ferricyanide and cupric ions of this medium competitively, simultaneously giving cupric ferrocyanide (Karnovsky's precipitate) and cuprous thiocholine iodide (Koelle's precipitate) as the histochemical products.[66] Tsuji and Larabi modified the method to promote the "true" Karnovsky's reaction and to slow down the secondary Koelle's reaction by increasing the concentration of the ferricyanide ion and decreasing the concentration of the cupric ion. ATCh chloride was used as a substrate instead of ATCh iodide to completely suppress the residual Koelle's precipitate. This is possible since the chloride salt of cuprous thiocholine is soluble unlike the iodide salt. The pH of the medium was also lowered from 6.0 to 5.0 to avoid artifactual nuclear staining, which appears at pH's over 5.5. Here, Karnovsky's cupric ferrocyanide becomes the sole precipitate at the enzymatic site, providing fine localization of AChE.

A commercial assay produced by ImmunoChemistry Technologies employs a completely different approach to directly quantify and localize active ChE enzymes in whole living cells. Their unique kit does not use antibodies—instead it is based on physostigmine, a known ChE inhibitor, which is linked to fluorescein, a green fluorescent label.[67] The fluorochrome-tagged physostigmine (Ph-F) is cell permeant, so it does not have to lyse the cells or permeabilize the membranes. There is no cross-reaction with inactive or proforms of the enzyme because, as mentioned above, this kit does not use antibodies. Only active ChE enzymes bind to Ph-F. They retain it on or within the cell, thus generating a green fluorescent signal.

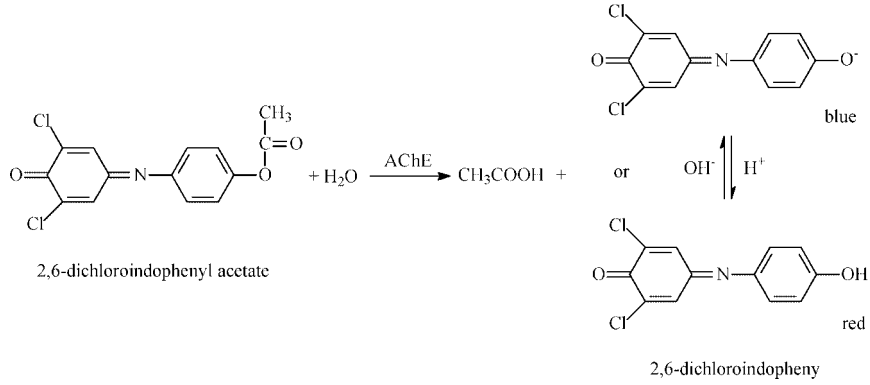
3.3. Colorimetric Sticks or Strips

The increasing public concern about pesticide contamination in food and the environment has increased the demand for broader and stricter pesticide monitoring. However, common laboratory methods such as gas or liquid chromatography are often time-consuming, labor-intensive, and expensive. They are poorly suited for analyzing large volumes of samples. The assays using colorimetric strips or papers to detect ChE inhibition by color development with the naked eye allow qualitative and semiquantitative detection of pesticides in the field.

Many of the early assays for detecting ChE activity were based on the enzymatic release of acetic acid and the resulting change in pH.[68] Bach et al. assessed a diagnostic dipstick produced by Merck (Darmstadt, West Germany) for assaying BChE activity.[69] In this assay, filter paper impregnated with a pH indicator and acetylcholine changes color with the amount of released acetic acid. The method has been adapted so that the color scale is graduated to give the enzyme activity. It takes only 6 min to complete. In another report, the strips of filter paper turn pink-to-violet in positive tests and yellow in negative tests. Here, phenol red was used as the indicator, and 1,2-propyleneglycol was used as the inhibitor of bacterial decomposition and as a wetting agent.[70]

A ChE-based dipstick-type assay for the class-specific detection of OP and carbamate pesticides has also been developed.[71] The proposed assay involves incubation of an AChE-coated strip in the pesticide solution followed by incubation of the sample-treated strip in a chromogenic substrate solution. The color intensity is monitored by the naked eye or a reflectometer. Nitrocellulose membranes, Immobilon (PVDF), Biodyne B, Hybond N^+, Immunodyne

Scheme 5. Catalytized Hydrolysis of 2,6-Dichloroindophenyl Acetate by AChE



ABC, and Ultrabind membrane were all tested for suitability of the immobilizing enzyme. Hybond N^+ proved the most suitable. The chromogenic AChE substrates tested were indophenyl acetate, 2,6-dichloroindophenyl acetate (Scheme 5), indoxyl acetate (Scheme 4), and *N*-methylindoxyl acetate, among which indophenyl acetate gave the best results.

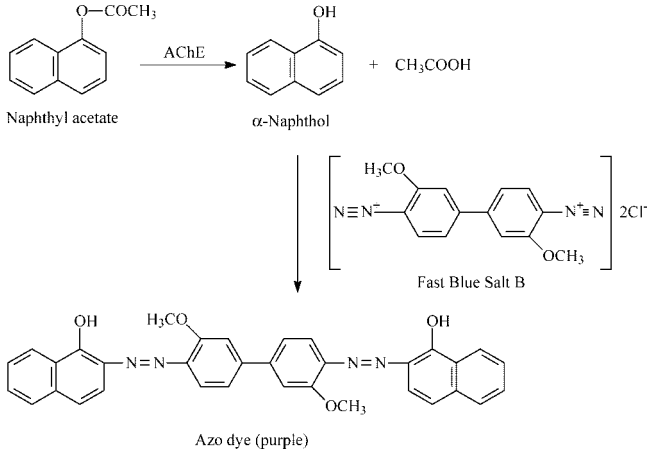
Among the currently available commercial products are OP Stick Sensor (Protein-Biosensor, Toulouse cedex, France) and Eclox-Pesticide Strips (Severn Trent Services, Colmar, Fort Washington, PA). The time and cost saved by these approaches may in fact be enormous.

3.4. Thin-Layer Chromatography (TLC) Assays

Thin-layer chromatography-based assays were developed because this technique gives information quickly about the activity localization of ChE inhibited by complex inhibitors. The separated constituents can be directly detected on the TLC plate both qualitatively and semiquantitatively.

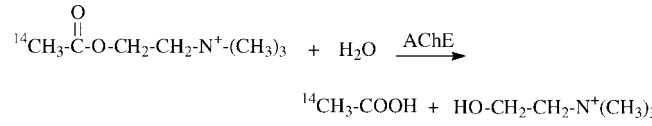
A simple and rapid bioautographic enzyme assay on TLC plates has been developed for the screening of possible ChE inhibitors from plant extracts (Scheme 6, Figure 6).[72] First, TLC plates were eluted with acetone or isopropanol and then thoroughly dried. After migration of the sample in a suitable solvent, the TLC plate was dried for complete removal of solvent. The plate was then sprayed with enzyme solution and thoroughly dried again. The naphthyl acetate solution and the Fast Blue B salt solution were mixed and sprayed onto the plate to give a purple coloration due to the formation of purple-colored diazonium dye after 1−2 min. In the presence of inhibitors, white spots were observed on the dye-colored background of the TLC plates.

Scheme 6. Reaction of AChE with Naphthyl Acetate and the Subsequent Formation of the Purple Dye in the TLC Bioassay[72]



3.5. Radiometric Assays

The radiometric assay of ChE has been reported only on rare occasions. A method for the microdetermination of AChE activity was developed in which the formation of C^{14} labeled acetic acid liberated enzymatically from the labeled substrate, was measured using a counting technique:[73]



One advantage of this method is that it can be applied to very small samples of tissue at relatively constant pH. Samples of the order of 1 μL of whole blood, blood plasma, or insect tissue deposited on cavity-type microscope slides have all been examined.[74,75]

Another radiometric assay for ChE in which an organic/water extraction separates ^{3}H-labeled acetylcholine from its products was reported by Johnson and Russell.[76] (^{3}H-acetyl)choline is enzymatically hydrolyzed in a small reaction volume in a scintillation vial. The released [^{3}H]acetate is then extracted into a toluene-based scintillator. The extracted [^{3}H]acetate can be counted efficiently, but the unhydrolyzed [^{3}H]acetylcholine remains unextracted in the small aqueous reaction volume. Its weak β-particles of decay cannot escape from the reaction medium to excite the scintillator. The assay is highly reproducible, quite sensitive, and useful for quick assay applications of multiple samples.

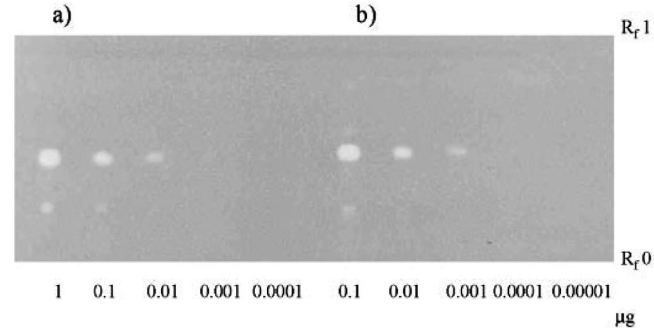


Figure 6. Bioautograph showing the inhibiton of AChE activity by (a) galanthamine and (b) physostigmine with different concentrations. Reproduced with permission from ref 72. Copyright Wiley-Blackwell.

The advantages of a radiometric assay are simplicity, reproducibility, and accuracy, as well as its extreme sensitivity.[77,78] In addition, they use natural substrates, which is different from most other assay methods. However, they are expensive and have a disposal problem due to the radioactive waste.

3.6. Calorimetric Assays

All of the current assay techniques for ChE activity measure some secondary reaction such as a pH or color change where the enzyme reaction itself is not measured.[79] However, a calorimetric assay has been developed that measures the heat of the reaction itself.[80,81] ChE activity in human serum was also measured calorimetrically by O'Farrell et al.[79] The response of the calorimeter when the serum is mixed with acetylcholine corresponds to the serum ChE activity. The thermogram peak is linearly related to enzyme activity at serum dilution volumes where heat interference was negligible. As with radiometric assays, one of the advantages of calorimetric assays is the use of natural substrates.

3.7. Biosensors

The bioanalytical detection of OP, carbamate pesticides, and various inhibitors using ChE, either in its free form or in solution or immobilized as a biorecognition element in biosensors, has a long tradition. Biosensors allow the use of ChE in combination with a variety of transducers, either electrochemical, optical, or piezoelectric. Because of their excellent performance at high specificity and sensitivity, rapid response, low cost, and relatively compact size, biosensors have become an important tool for clinical, food, and environmental monitoring.

3.7.1. Electrochemical Biosensors

Electrochemical analysis affords many advantages including high sensitivity, simple sample treatment protocols, inexpensive instruments, and easy operation procedure. It is one of the most widely applied detection techniques in biosensor systems and includes conductometric, potentiometric, and amperometric transducers.

3.7.1.1. Conductometric Biosensors. Conductivity is a measure of the ability of solution to pass an electrical current, and it is directly proportional to the concentration of ions in the solution. A conductometric transducer, i.e., a conductivity meter, was employed to detect the activity of ChE catalyzing the hydrolysis of acetylcholine into charged products, thus increasing the conductivity of the solution:[82]

$$\text{acetylcholine} + H_2O \xrightarrow{\text{AChE}} \text{choline} + CH_3COO^- + H^+$$

The device was designed specifically to contain the offset and the amplification circuits that enable the measurement of the small conductivity change caused by the enzymatic reaction. A preliminary study of such biosensors indicated that this conductivity meter can be used as a conductometric transducer in biosensor systems.

One advantage of conductometric biosensors is that they do not require any reference electrode and the conductometric transducer can be miniatured with a two-electrode system. An interdigitated structure is preferable for the development of conductometric electrodes.[83]

3.7.1.2. Potentiometric Biosensors. ChE activity can also be followed by a potentiometric electrode. The Michel method, first described in 1949, has been one of the most popular methods for the routine determination of blood ChE.[84] In this method, ChE is allowed to catalyze the hydrolysis of acetylcholine for 1 h. The enzyme activity is represented as the rate of pH change (ΔpH/h) as a result of the released acetic acid. Because of their relative simplicity, the procedure first described by Michel and the many that followed with more or less significant modifications have often been used in field studies.

However, the accuracy and the sensitivity of the Michel method are not as great as those of the titrimetric method. This is primarily because the former measures the pH, which is a logarithmic function of the acid concentration, rather than the acid production itself.[85] The acid released from the enzymatic hydrolysis of acetylcholine can be monitored by titration with standard alkali at constant pH using either an indicator or a potentiometer. The automated pH-stat method has proven to be one of the most convenient methods available for assaying ChE in which the acid liberation can be continuously recorded. The pH-stat method offers better control of the assay conditions, resulting in higher sensitivity and better reproducibility.[86] However, it is less suitable for field work because of its complexity.

Glass electrodes, ion-sensitive field-effect transistors (ISFETs), and other pH-sensitive potentiometers have all been employed to detect the pH change in enzymatic reactions.[87] A potentiometric AChE biosensor based on a pH-sensitive PVC membrane with a plasma-polymerized ethylenediamine film has been proposed.[88] The glow discharge plasma technique was utilized to deposit a film containing amino groups on the surface of a pH-sensitive poly(vinyl chloride) (PVC) membrane. Such an approach makes it possible to fabricate a very simple self-mounted AChE membrane directly attached to a PVC pH-sensing electrode surface. A comparison of dioctyloctadecylamine (DOODA) and *N*,*N*-didecylaminomethylbenzene (DAMAB) as the pH-carrier led to the conclusion that the DAMAB-based biosensor offered a better response performance.

The ISFET is a new type of miniaturized semiconductor chemical sensor, in which the normal metal−oxide−silicon field-effect transistor (MOSFET) gate electrode is replaced by an ion-sensitive surface with the ability to detect ion concentrations in solution. It opens up a new boundary area between electronics and biotechnology. Applications using this low-cost, disposable, and simple monitoring device have been widely developed since 1970.[89] Enzyme-modified ISFETs (ENFETs) are based on a pH shift caused by an enzymatic reaction. Langmuir−Blodgett (LB) films containing BChE are fabricated to produce an ENFET for the detection of OP pesticides.[90] Enzyme/stearylamine mixed LB films are modified onto an ISFET surface and treated by glutaraldehyde vapor to improve the LB film stability. The ENFET thus obtained worked as a potentiometric biosensor for trichlorfon detection on the basis of enzyme inhibition. In another report by Hai et al.,[91] AChE was immobilized onto the ISFET gate surface using cyanuric−chloride as the coupling molecule. The resulting ISFET biosensor was used to detect reagents that inhibit AChE activity. The catalytic activity of the immobilized AChE is inhibited in a reversible manner by eserine, a competitive and reversible inhibitor. ISFETs are very attractive candidates for the development of biosensors due to their fast response, high

sensitivity, batch processing capability, microsize, robustness, need for minimal maintenance, and potential for on-chip circuit integration.[92] A lot of research and development has been done on such enzyme-modified ISFETs during the past few years.

A most attractive new approach is the new trend to develop nanoscale ISFETs.[93] Tans et al. first demonstrated the possibility of using a semiconducting single-wall carbon nanotube (CNT) as a field-effect transistor.[94] The subsequent functionalization of CNT with bioreceptors leads to the development of various novel biosensors. Recombinant AChE was immobilized on the reverse side of the carbon nanotube sensor for detecting OP insecticides.[95] The current between the source and the drain was measured after incubation of various concentrations of OPs with immobilized AChE. Two OPs showed similar dose-response curves in measuring $I_{ds}-V_{gate}$ correlation with the AChE-immobilized CNT sensors.

The potentiometric biosensors have to be used in weakly buffered solutions, and hence, they often suffer from the interference of background ammonia or acid fumes. This is the inherent disadvantage of potentiometric biosensors. For accuracy, buffer solutions must be freshly prepared at a precise pH.

The pH shift owing to the release of the acid from acetylcholine hydrolysis can also be estimated by the change in color of an indicator rather than with a pH or potentiometric meter. A number of indicators have been used, including phenol red, *m*-nitrophenol, cresol red, bromocresol purple, litmus, bromothymol blue, etc.

3.7.1.3. Amperometric Biosensors. Amperometric biosensors are quite simple compared with potentiometric devices. They are widely employed for the development of various ChE-based biosensors.[96,97] An amperometric bienzyme sensor based on ChE and ChO has been constructed.[98] First, the ChE hydrolyzes acetylcholine to produce choline and acetate. Choline is not electrochemically active and, therefore, cannot be detected directly. Therefore, ChO is used to produce H_2O_2, which can then be detected amperometrically:

$$\text{choline} + 2O_2 + H_2O \xrightarrow{\text{ChO}} \text{betaine} + 2H_2O_2$$

This indirect detection of ChE activity by monitoring the oxidation current of H_2O_2 often suffers from interference from other electroactive species in the sample due to the high applied potential.[11]

Various chemically modified electrodes were developed to decrease the detection potential of H_2O_2. An amperometric biosensor based on the electrodeposition of gold−platinum bimetallic nanoparticles onto 3-aminopropyl triethoxy silane modified glassy carbon electrode for the detection of paraoxon ethyl, aldicarb, and sarin has been developed.[99] AChE and ChO were immobilized onto a modified electrode. The inhibition of AChE by pesticides was monitored by measuring the decrease in the oxidation current of H_2O_2 onto the electrode surface. The gold−platinum bimetallic nanoparticles showed excellent electrocatalytic activity for H_2O_2 at low applied potentials. Another option is to measure the consumption of oxygen in the ChO catalyzed reaction.[100,101]

If ATCh or BTCh is used as the substrate for the ChE enzymes, thiocholine is produced during the enzymatic reaction. Thiol-containing compounds are known to be oxidizable at the electrode surface, and the anodic oxidation current of thiocholine is inversely proportional to the concentration of the OPs in the sample:[102]

$$\underset{\text{thiocholine}}{2HSCH_2CH_2N^+(CH_3)_3} \xrightarrow{\text{anodic-oxidation}} \underset{\text{dithiobischoline}}{(CH_3)_3N^+CH_2CH_2S-SCH_2CH_2N^+(CH_3)_3} + 2H^+ + 2e^-$$

However, the amperometric measure of thiocholine has been difficult to achieve at common electrodes because of the high overpotential applied. Other electroactive species such as ascorbic acid and uric acid present in real samples may interfere with the detection signals. A method to circumvent this problem has been suggested. It is to use redox mediators, such as hexacyanoferrate, tetracyanoquinodimethane, tetrathiafulvalene, dimethyl ferrocene, cobalt phthalocyanine, etc. to lower the overpotential.[103−105] Pyrroloquinoline quinone has been entrapped in a polypyrrole matrix on a glassy carbon electrode to mediate the amperometric detection of thiocholine at 500 mV versus Ag/AgCl.[106] This method demonstrated the versatility of this particular sensor and its ability to rapidly and sensitively evaluate the enzymatic activity of AChE and its inhibition in the presence of the pesticide.

Meanwhile, Prussian blue and various nanomaterials such as precious metal, quantum dots, carbon nanotubes, etc. have been used to fabricate various chemically modified electrodes and biosensors.[107−110] Joshi et al. developed a disposable biosensor for the detection of OP insecticides. In their design, AChE and acid-purified multiwall carbon nanotubes were modified onto the thick filmstrip electrodes. The large surface area and electrocatalytic activity of the carbon nanotubes lowered the overpotential for thiocholine oxidation to 200 mV (vs Ag/AgCl), without the use of mediating redox species.[107]

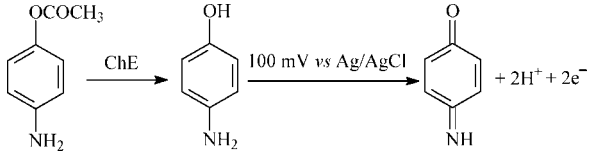
Thiol or disulfide derivatives can spontaneously form a closely packed and ordered monolayer (self-assembled monolayer, SAM) on a metal surface. The SAM formed on the metal electrode desorbs in alkaline solution through a one-electron path. A method of adsorptive stripping voltammetry has also been successfully applied to the highly sensitive determination of AChE activity by the chemisorption/desorption process of thiol compounds.[111] The thiocholine released from the AChE-catalyzed hydrolysis of ATCh chemisorbed onto a silver electrode surface through incubation. Then, the silver electrode was transferred into an alkaline solution to electrochemically strip the adsorbed thiocholine through reductive desorption:

$$(CH_3)_3N^+CH_2CH_2SH + Ag \rightarrow (CH_3)_3N^+CH_2CH_2SH-Ag + 1/2H_2$$

$$(CH_3)_3N^+CH_2CH_2SH-Ag + e^- \rightarrow (CH_3)_3N^+CH_2CH_2S^- + Ag$$

Apart from the use of a classical thiol ester as substrate, other substrates capable of giving electroactive products were also explored. La Rosa et al. described a monoenzymatic ChE biosensor that uses 4-aminophenyl acetate as the substrate.[112] The hydrolysized product of the reaction from

4-aminophenyl acetate by ChE catalysis is 4-aminophenol, which can be measured amperometrically:



The advantages of this substrate are simplicity of the measurement procedure and low applied potential. However, the spontaneous oxidation of 4-aminophenyl acetate to *p*-aminophenol decreases its performance and applicability.

In general, low detection limits have been achieved by the use of highly sensitive genetically engineered enzymes. However, Sotiropoulou and Chaniotakis also achieved it using a nanoporous carbon matrix.[113] On the one hand, the activated carbon is able to selectively concentrate the pesticide, while on the other hand, the nanostructure of the carbon aids in the physical confinement of the AChE inside relatively small cages against unfolding. In this way, the stability of the AChE is improved, thus providing biosensors with higher operational and storage stabilities.

3.7.1.4. Square-Wave Voltammetry. A square wave voltammetry (SWV) method was developed for the assessment of the impact of OP compounds on the ChE of *Pheretima* with 2,6-dichloroindophenol (2,6-DCIP) as a redox indicator.[114] The thiocholine (RSH) produced from the AChE hydrolysis of ATCh reacts with 2,6-DCIP, a blue colored compound, subsequently reduced to a colorless product 2,6-DCIP$_{Re}$. At the same time, RSH is oxidized to RSSR. In addition, the thiolate can attack the H atom of the conjugate phenol ring of the 2,6-DCIP, causing a side reaction. The 2,6-DCIP−SR$_{Ox}$ produced gives a new electrochemical signal:

$$\text{2,6-DCIP}_{Ox} + 2H^+ + 2e^- \overset{\text{electrode}}{\longleftrightarrow} \text{2,6-DCIP}_{Re}$$

$$\text{2,6-DCIP}_{Ox} + \text{RSH} \rightarrow \text{2,6-DCIP}-\text{SR}_{Re} + \text{RSSR}$$

$$\text{2,6-DCIP}_{Re} + \text{RSH} \rightarrow \text{2,6-DCIP}-\text{SR}_{Re}$$

$$\text{2,6-DCIP}-\text{SR}_{Re} - 2H^+ - 2e^- \overset{\text{electrode}}{\longleftrightarrow} \text{2,6-DCIP}-\text{SR}_{Ox}$$

The reduction peak of 2,6-DCIP was observed from SWV studies using a negative scan. It decreases with the enzymatic reaction time. The decrease rate of the 2,6-DCIP reduction peak is expressed as the ChE activity.

SWV gives more advantages over common amperometric or potentiometric assays. On the one hand, because it uses a pulsed waveform, the detection sensitivity is enhanced by the repeated oxidation and reduction of the same analyte species. On the other hand, limiting currents from dissolved species such as oxygen do not interfere with the analytical signal since SWV is a purely subtractive technique with discrimination against background currents. In addition, SWV is a fast technique, with the voltammogram obtained in a matter of seconds.

3.7.2. Optical Biosensors

Most of the biosensors discussed above have been developed using electrochemical transducers. However, since optical sensors are not interfered with by electric fields, it is easy to develop multiple assaying sensors. In addition, optical sensors using optical fibers have the advantage of being useful in remote sensing applications.

An optical biosensor consisting of immobilized Nile Blue (ETH5294) (CM) and AChE was employed to detect dichlorvos.[115] The highly lipophilic nature of the chromoionophore CM and its compatibility with the sol−gel matrix prevented any leaching. This is frequently a problem in optical sensors constructed using pH indicator dyes. The CM layer is pH-sensitive and detects pH changes due to AChE catalysis. In another report, AChE and a bromothymol blue (BTB) doped sol−gel film were used to develop an optical fiber biosensor for chlorpyrifos.[116] The biosensing element on the single sol−gel film was placed inside a flow-cell in order to produce a flow system. A color change of the BTB and the measured reflected signal at wavelength 622 nm are related to the chlorpyrifos concentration in the samples. Ozturk et al. developed a series of reversible biosensors based on 2-(3,5-dinitrophenyl)-4-[4-(1,4,7,10-tetraoxa-13- azacyclopentadecyl)benzylidene]-5-oxazolone (CPO-I), 2-(4-nitrophenyl)-4-[4-(1,4,7,10-tetraoxa-13-azacyclopentadecyl)benzylidene]-5-oxazolone (CPO-II), and 2-(4-tolyl)-4-[4-(1,4,7,10-tetraoxa-13-azacyclopentadecyl)-benzylidene]-5-oxazolone (CPO-III) derivatives immobilized in a polyvinylchloride matrix doped with AChE.[117] The CPO-III sensor membranes were more sensitive to acetylcholine than either CPO-I or CPO-II due to electron enrichment of the molecule caused by the *o*-tolyl group. These fluorescent CPO derivatives can be used as alternative indicators for enzymatic neurotransmitter and donepezil sensing. A fiber-optic biosensor consisting of an AChE-immobilized LB film was developed for the detection of OP compounds in contaminated water.[118] AChE molecules were adsorbed onto a viologen monolayer through electrostatic forces. The sensing scheme, due to OP inhibition on AChE, was based on the decrease of the yellow product, *o*-nitrophenol, produced from *o*-nitrophenyl acetate, the colorless substrate.

An interesting liposome-based fluorescent biosensor has been described for the detection of OP pesticides.[119] AChE and the pH-sensitive fluorescent indicator pyranine were immobilized in nanosized liposomes, which provide a suitable environment for the effective stabilization of AChE. Porins, embedded within the lipid membrane, allow the free transport of both the substrate and the pesticide in and out of the liposomes. The decrease in the fluorescent signal of the pH indicator is relative to the concentration of dichlorvos and paraoxon. This biosensor has been successfully applied to the detection of the total toxicity in drinking water samples.[119]

A novel optical sensor for detecting anticholinesterases was constructed by immobilizing fluorescein isothiocyanate (FITC)-tagged AChE on quartz fibers and monitoring the enzyme activity.[120] The pH-dependent fluorescent signal was quenched by the protons produced during acetylcholine hydrolysis by FITC-AChE. Edrophonium reversibly inhibited AChE and consequently reduced fluorescence quenching. Once the inhibitor was removed, the biosensor response immediately recovered. The carbamate neostigmine also inhibited the biosensor response, but recovery was much slower. Since this instrument is portable, it has potential for adaption for field use.

The excitation of surface plasmons by light is known as surface plasmon resonance (SPR) for planar surfaces.

Because of its high sensitivity, SPR technique has been widely applied for the quantitative detection of various chemical and biological compounds. A SPR-based fiber-optic sensor for the detection of chlorpyrifos has been reported.[121] The probe is prepared by immobilizing AChE on the silver-coated core of a plastic-coated silica fiber. Detection is based on the competitive binding of the pesticide for the substrate ATCh to AChE. The SPR wavelength decreases with the increase in the pesticide concentration in the fluid around the probe.

The excitation of surface plasmons by light for nanometer-sized metallic structures is known as localized surface plasmon resonance (LSPR). The resonance frequency of the LSPR is highly dependent on the local environment of the nanometer-sized metallic structures. Therefore, their absorbance is sensitive to the refractive index of the surrounding solvent as well as the binding events to those functionalized nanostructures. Lin et al. applied the LSPR of gold nanoparticles coupled with AChE to create a biosensor for detecting OP agents.[122] A self-assembled gold nanoparticle monolayer was first coated onto the uncoated portion of an optical fiber. AChE was then immobilized by covalent coupling onto this gold nanoparticle layer. The capture or reaction of acetylcholine by the immobilized AChE should cause a local increase in the refractive index, leading to a decrease in light intensity. Pesticides inhibited the AChE activity, leading to a change in light attenuation. The AChE-modified LSPR biosensor can also be used for label-free detection of paraoxon.

White et al. presented an evanescent wave technique for a rapid reagentless assay for competitive inhibitors of AChE and BChE. This technique allows for the discrimination between three groups of inhibitors: those which inhibit AChE, those which inhibit BChE, and those which inhibit both.[123] Monosulfonate tetraphenyl porphyrin (TPPS1) is a reversible, competitive inhibitor for both AChE and BChE. The interaction of TPPS1 with immobilized AChE and BChE exhibits unique spectral characteristics, yielding characteristic absorbance peaks at 446 and 421 nm, respectively. Exposure of the immobilized TPPS1-enzyme to competitive inhibitors of either enzyme results in a loss in absorbance of the characteristic peak. Noncompetitive inhibitors have no effect on absorbance. Exposure to inhibitors of both enzymes results in loss of absorbance intensity of both porphyrin-enzyme peaks.

Chemiluminescence (CL) has become an attractive analytical tool in pesticide determination in recent years due to its high sensitivity, rapid assay, and inexpensive instrumentation. Godoy et al. immobilized AChE at the surface of a miniaturized biomimetic nanostructure to develop an electrochemiluminescent (ECL) device.[124] In their study, AChE and ChO are combined to catalyze acetylcholine and ultimately produce H_2O_2, which is finally involved in the ECL reaction of luminol. This gives rise to a light signal proportional to both choline and acetylcholine concentrations. Lin and Chen reported that pirimicarb, methomyl, aldicarb, and carbofuran could greatly enhance the ECL intensity of $Ru(bpy)_3^{2+}$ at a MWNT/Nafion-modified glass carbon electrode.[125] $Ru(bpy)_3^{2+}$ was first oxidized to produce $Ru(bpy)_3^{3+}$ at the electrode. The carbamate (C−N) loses a proton to give the strongly reducing intermediate C−N*, which then further reacts with $Ru(bpy)_3^{2+}$ to form $Ru(bpy)^{3+}$. $Ru(bpy)^{3+}$ in turn reacts with $Ru(bpy)_3^{3+}$ to yield $Ru(bpy)_3^{2+*}$. When $Ru(bpy)_3^{2+*}$ reverses to give $Ru(bpy)_3^{2+}$, light is emitted.

3.7.3. Photothermal Biosensors

Thermal lens spectrometry (TLS) is one of a group known as photothermal techniques. A photothermal AChE biosensor was developed by Pogačnik and Franko with a view to achieving low detection limits and relatively short assay time.[126] AChE was immobilized into glass beads with controlled pore sizes. These were then packed into a PEEK (polyetheretherketone) column. The activity (a) of AChE is related to the thermal lens signal caused by the injected substrate. This technique relies upon the absorption of optical radiation by a laser in the sample and the subsequent nonradiative relaxation of absorbed energy to produce heat. This then yields a detectable change in the refractive index of the sample because of the increase in the sample temperature. The remaining relative enzyme activity was calculated according to the ratio: a_f/a_0, where a_0 is the initial AChE activity and a_f is the inhibited AChE activity. The results for carbofuran, propamocarb, oxidemeton/methyl, and parathion/ethyl all showed sufficient sensitivities, in good agreement with standard GC-MS detection methods.

3.7.4. Quartz Crystal Microbalance Biosensors

The quartz crystal microbalance (QCM), as a mass-sensitive detector, has been widely used in the development of biosensors. The sensitivity of a QCM-based enzyme sensor can be amplified significantly by exploiting the precipitation of enzymatic reaction products to increase the mass deposition. AChE, ChO, and HRP were assembled on either Au electrodes or Au-quartz crystals to sense acetylcholine. The resulting insoluble product on the transducers by the HRP-mediated oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) correlates with the concentration of acetylcholine.[127]

Another biosensor based on the quartz crystal microbalance was developed for the determination of OP and carbamate pesticides.[128] Exposure of the immobilized enzyme to a substrate solution of 3-indolyl acetate produced an insoluble indigo pigment product accumulating on the crystal surface. The enzyme activity can be followed in real time by measuring the frequency changes associated with the mass changes at the crystal surface. The presence of paroxon or carbaryl is detected by a decrease in the frequency changes due to their inhibitory effects on the enzyme.

A new method was introduced for the sensitive detection of ChE inhibitors based on real-time monitoring using a piezoelectric biosensor.[129] First, the conjugate of *N*-mercaptoundecanoic acid (MUA) with *N*α,*N*α-bis(carboxymethyl)-L-lysine (NTA-Lys) was self-assembled on the surface of a gold electrode. Then, a paraoxon-spacer-hexahistidine conjugate was linked to the MUA−NTA-Lys layer via the chelate complex with Ni^{2+}. The paraoxon-modified surface was used as the recognition element for BChE. In the presence of other free inhibitors, the binding of BChE to the immobilized paraoxon decreases. In this way, a competitive affinity assay for OP compounds was developed.

3.8. Chip Techniques

A microfabricated device for flow injection analysis and electrophoretic separation of AChE inhibitors has been described.[130] Solutions of inhibitor, enzyme, substrate, and derivitizing agent were mixed within the microchip channels using computer-controlled electrokinetic transport. The thiocholine released from the AChE-catalyzed hydrolysis was

then reacted with coumarinylphenylmaleimide to form a thioether, which was detected by laser-induced fluorescence. Inhibitors reduced the decrease of fluorescence signal. The potential of this technique as a multiplex screening device is illustrated by the result where a mixture of four cationic inhibitors, tacrine, edrophonium, and tetramethyl- and tetraethylammonium chloride, was separated and detected in 70 s.

The next generation of biosensors involves exciting chip techniques: lab-on-a-chip or micro total analysis systems (μTAS). They integrate many laboratory functions on a single chip of only a few millimeters to a few square centimeters. They are capable of dealing with the handling of extremely small fluid volumes down to less than picoliters. They can be used to carry out full-scale analyses from sample introduction to chemical separation and detection, on a single, miniaturized device. Tan et al. reported a lab-on-a-chip device for detecting sarin in a small volume of blood.[131] The device was fabricated in polymethylmethacrylate using CO_2-laser micromachining. Many functions were integrated within the single chip by continuous-flow microfluidics. These include whole-blood lysis, regeneration of free sarin from its complex with blood ChE, protein precipitation, filtration, enzyme-assisted reactions, as well as optical detection. This example demonstrates the potential use of a handheld device for the field use of suspected nerve agents.

4. Important Role Played by Organic Solvents

Most OP toxicants are generally characterized by their low solubility in water and a concomitant high solubility in organic solvents. However, enzymatic catalysis has been traditionally performed in aqueous solution. Therefore, the use of enzymes capable of also functioning in the presence of organic solvents would open up a far wider range of applications. ChE can usually function in a manner similar to aqueous solution in low concentrations of organic solvent in a mixed aqueous−organic phase.[132,133] To circumvent the problem of hydrophilic solvents stripping the enzymes of essential water, it was suggested that 1−10% water was necessary for enzymatic activity and should be added to the organic solvent for sufficient hydration of the active site of the enzyme.[134] However, a "bienzymatic inhibition organic-phase enzyme electrode" has been developed that can be used to check either OP pesticides or carbamates. It uses a water-saturated chloroform−hexane mixture (50%, v/v) as the solvent. The percentages of water in both hexane and chloroform, even when water-saturated, are always <0.1% (w/w).[135] Both BChE and ChO were immobilized in κ-carrageenan gel, and an amperometric gas diffusion electrode for oxygen was used.

A disposable ChE biosensor based on screen-printed electrodes was used to assess the effect of miscible organic solvents on AChE activity and on the inhibitory effect of OP pesticides.[136] Acetonitrile and ethanol, in concentrations ranging between 5 and 15%, showed an activating effect on the ChE. Pure acetonitrile inhibited 50% of enzymatic activity. Ethanol and dimethylsulfoxide (DMSO) fully inhibited ChE. Acetonitrile (5%) and 0.2% polyethylenimine were found not to interfere with the enzyme−inhibitor interactions. Reported by Valdés-Ramírez et al., amperometric AChE biosensors were developed for the quantification of carbofuran, carbaryl, methylparaoxon, and dichlorvos in a phosphate buffer containing 5% acetonitrile.[104] AChE was immobilized onto cobalt(II) phthalocyanine-modified electrodes by entrapment in a photo-cross-linkable polymer (PVA-AWP).

Dondoi et al. presented an analytical method for OP insecticides based on AChE biosensors coupled with preconcentration and oxidation on a solid-phase column.[137] Acetonitrile, ethanol, and methanol were all tested for their influence on AChE activity, insecticide inhibition, and their ability to elute the adsorbed insecticides. The presence of the organic solvent in the incubation media was found to decrease the inhibition percentages: a 15% organic solvent level induced an important reduction of the inhibition. The negative effect of the organic solvent on the enzyme increased with incubation time. Ethanol had the smallest denaturing effect followed by methanol and acetonitrile. As a compromise, since 5% acetonitrile had the least negative effect on the biosensor analysis, it was chosen as the most appropriate organic solvent for column elution.

An AChE-based amperometric bioelectrode was reported for measuring enzyme activity with paraoxon dissolved in organic solvents.[138] The inhibition percentage induced by a paraoxon in an organic solvent increases in the following sequence: acetonitrile < water < hexane. A synergetic effect of inhibition by paraoxon and a pseudoinhibition by organic solvents induced a mixed inhibition of noncompetitive/uncompetitive mechanisms.

Fennouh et al. studied the inactivation of both free and immobilized AChE by paraoxon in the presence of different organic solvents using ChO immobilized on an oxygen electrode.[139] Enzyme inhibition by paraoxon was measured in the presence of various organic solvents within the concentration range 0.0−10%. Free AChE was found to be more sensitive to paraxon inhibition than immobilized AChE. Among the miscible and immiscible organic solvents tested, acetone and cyclohexane, respectively, allowed the highest enzyme inactivation by paraoxon on free AChE. 0.05% acetone inactivates 42% of the free AChE activity by paraoxon. Whereas 5% cyclohexane enhances the paraoxon inhibition efficiency with a 250-fold improvement compared to no organic solvent, in 0.05% acetone, a 35-fold effect was observed.

Although the residual AChE activity could also be measured in a buffer solution after evaporation of the solvent containing the pesticides, the time required for the overall test procedure was extended. To overcome this, an extraction of the pesticide from food samples using isooctane is performed.[140] The results show that an incubation time of 30 min of the AChE-biosensor in isooctane caused only a marginal decrease of 3% in enzyme activity. Using isooctane as the extraction solvent yielded an 84% recovery of paraoxon from orange juice.

ChEs are irreversibly inhibited by OP and carbamate pesticides and reversibly inhibited by heavy metals. Hence, when evaluating pollution levels using AChE activity, heavy metals in the sample could negatively affect the assay results.[141] In addition, an overestimation of pesticide level due to the thiocholine produced by the enzymatic reaction binding with heavy metals due to the high affinity of thiols for heavy metals could result. A novel extraction method was developed to avoid the interference of heavy metals and other water-soluble enzymatic inhibitors such as fluoride and hyplochlorite, which have a low solubility in organic solvents.[141] First, two immiscible phases were formed. The pesticide is solubilized in the organic phase, the enzyme is solubilized in the aqueous phase, and AChE irreversibly

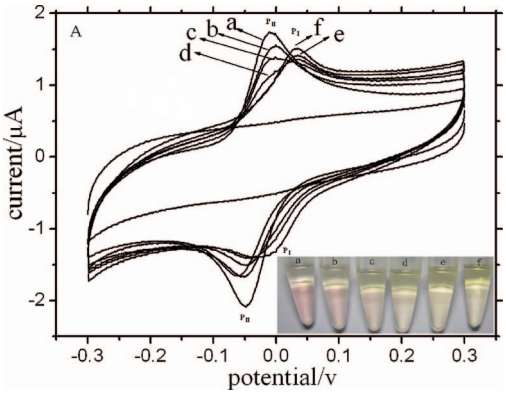
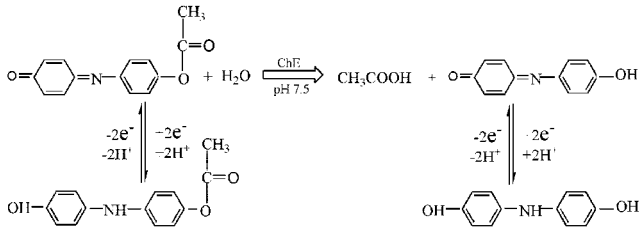


Figure 7. CV of glass carbon electrodes in water phase after 20 min enzymatic reaction at the interface of two phases. The ChE was diluted in pH 7.5 PBS with the fold of 1 (a), 2.5 (b), 5 (c), 10 (d), 20 (e), and 50 (f), respectively. Reproduced with permission from ref 142. Copyright Elsevier.

Scheme 7. Hydrolysis of Indophenol Acetate to Produce Indophenol by ChE and Electrochemical Reaction of Both of Them[142]



binds the pesticide at the aqueous/organic interface, resulting in an inhibition of the enzyme. The best results in terms of residual activity (100%) were when hexane with 10 min incubation period was used. This indicates that an extremely low solubility is preferred for the organic solvent because of its less negative effect on enzyme activity. In this way, the interferences from heavy metals or other water-soluble enzyme inhibitors are largely avoided.

Miao et al. studied the SWV assay of OP inhibition on ChE using two immiscible phases with the substrate and methyl parathion in the isooctane as the organic phase and the enzyme in the aqueous phase.[142] Water-insoluble indophenol acetate (yellow) was hydrolyzed by ChE at the interface of the two phases to produce the water-soluble indophenol (red). The latter spontaneously penetrates into the aqueous solution, giving rise to both the color change and the electrochemical signal (Figure 7). The ChE activity is related to the decrease in the indophenol acetate substrate and the increase of indophenol product and can be expressed by the peak current ratio of Ip_{II}/Ip_I, where Ip_{II} is the current value of indophenol and Ip_I is that of the indophenol acetate (Scheme 7). Methyl parathion in the isooctane phase inhibited the ChE activity at the interface of the two phases. The inhibition of pesticide was evaluated by determining the decrease in Ip_{II}/Ip_I.

The development of organic-phase enzyme assays has attracted considerable interest.[132,133] It made it possible to extend this analytical field to hydrophobic substrates and inhibitors with decreased interference arising from hydrophilic species. Furthermore, it improves the working life of ChE due to the lower levels of microbial contamination and the higher thermal stability of ChE in organic solvents because chemical and structural changes of enzymes require the existence of water.

5. Assaying ChE Activity and Its Inhibition: Applications

ChE activity and inhibition assays are also used in a wide range of applications: to diagnose clinical diseases, to assess the nature and extent of human, domestic animal, and wildlife exposure to OPs and carbamates, or as biomarkers to assess the environmental impact of pesticides. There is also the possibility of using such assays to estimate the concentration of OPs and carbamates in food or environmental samples. Inhibitor screening is also useful in the ongoing search for new drugs or pesticides.[91]

5.1. Clinical Diagnosis

AChEs are related to human memory, Alzheimer's disease, and many neurological disorders. The presence of AChE in amniotic fluid can be tested for in early pregnancy. Elevation of plasma BChE was also observed in acute myocardial infarction. The hepatocirrhosis compensation period and liver cancer can also both reduce enzymatic activity.[1]

In order to measure AChE activity alone, BChE activity needs to be inhibited or eliminated. In many literature reports, authors report AChE measurements in human plasma with acetylcholine as substrate, but in fact there is considerable confusion regarding the details.[5] Human plasma contains <2% of true AChE. In fact, human blood contains five different esterases, all of which take part in the hydrolysis of various substrates: AChE, BChE, two aromatic esterases (one present in red cells and the other present in plasma), and an aliphatic esterase found only in red cells. In general, the indoxyl esters and x-naphthyl acetate are hydrolyzed at far faster rates by the two ChEs than by the aliphatic and aromatic esterases. The hydrolysis of indoxyl butyrate by red cells is an exception.[143]

Both OP and carbamate pesticides irreversibly inhibit the ChE activity. This leads to the accumulation of acetylcholine, which can interfere with muscular responses and induce symptoms ranging from increased salivation and headaches to convulsions and suppressed breathing, eventually leading to death. Hence, ChE assays are useful for assessing the nature and extent of of human, wildlife, and domestic animal exposure to both OPs and carbamates. The primary toxic effect of them is AChE inhibition. However, many compounds inhibit BChE at even higher rates than AChE, in which cases BChE is a better indicator.[144] In clinical biochemistry, BChE determination in plasma or serum is more frequently used than that for AChE in red blood cells. Because there is no general rule for predicting which particular enzyme will be more inhibited by a given compound, it is suggested that the activity of both ChEs be measured in human blood to more reliably assess the effect of the inhibitors. Use of whole-blood samples is preferred since it eliminates the need to separate erythrocytes and plasma. Use of whole blood has also been recommended as a guideline for the international standardization of ChE measurements.[145] It has been suggested that whole-blood ChE monitoring should be adequate for livestock species such as cattle, horses, and sheep in which 90% of the total ChE activity is in the red blood cells. Other investigators developed a protocol for biomonitoring exposure in livestock to OP nerve agents. According to their protocol, exposure to ChE-inhibiting compounds should be suspected when the blood ChE activity in a sentinel group is >20% below the individual baseline ChE activity.

5.2. ChE Biomarkers for the Assessment of Environmental Impact

Both OP and carbamate pesticides are widely used in agriculture. However, they are also highly toxic to nontarget species, often causing environmental impact through air, water, soil, and food contamination. These compounds produce acute toxicity by inhibiting ChE in both invertebrate and vertebrate organisms: this leads to weakness, convulsions, paralysis, and even death The localization and classification of ChEs in invertebrates and vertebrates is broad, with various forms present in different tissue types.

Although the pesticides concerned are relatively volatile and are rapidly hydrolyzed under environmental conditions, their inhibitory effects can persist. ChE activity and its inhibition of various organisms have all been intensively explored as a possible biomarker for environmental pollution to assess their impact on cropland, forestry, and aquatic habitats. Reports include soil life such as carabid beetles, daphnia, earthworms, frogs, and rats,[146−150] aquatic life such as fish, prawns, mussels, and river snails,[151−159] and various wild life such as poultry and livestock.[47,160] In the study of Oropesa et al., the ChE activity of seabirds was determined in order to assess the environmental impact of spilled fuel containing high concentrations of polycyclic aromatic hydrocarbons from the tanker Prestige in 2002.[161]

5.3. Determination of ChE Inhibitors

As discussed above, OP, carbamate pesticides, and many other toxic compounds inhibit the activity of ChE. Hence, they can be used to determine the concentration of such compounds from environmental, food, agricultural, and aquatic product samples.[162−165] Among the most commonly used pesticides are diazinon, dichlorvos, malathion, parathion, and paraoxon. They all contain a phosphonate group mimicking the acetate moiety of acetylcholine as well as in many cases a positively charged group, linked by an ester or thioester linkage, mimicking the choline moiety. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) are commonly used techniques for determining the concentration of OPs. However, they are either expensive or time-consuming due to complicated sample preparation and preconcentration requirements. Also, because sophisticated equipment is usually needed, in-field detection appears impractical.

Class-specific determination of pesticides prior to chromatographic analyses could be an attractive approach for broader pesticide monitoring.[71] Recently, there have been numerous reports of the development of OP and carbamate pesticide class-specific determination based on ChE inhibition. Among the various assay methods based on ChE inhibition that have been developed using either optical or electrochemical transducers, or color dipstick techniques, are the many portable devices for on-site monitoring.

However, some attempts have also been made to detect different components simultaneously. An optical biosensor consisting of glutathione S-transferases (GST) and an AChE-immobilized gel film was developed to detect captan and OP compounds simultaneously in contaminated water.[166] The sensing scheme was based on the measurement of (s-(2,4-dinitrobenzene) glutathione and a-naphthol product formation by GST and AChE. The absorbance of s-(2,4-dinitrobenzene) glutathione and a-naphthol was detected by the optical biosensor system. It was observed that AChE was inhibited by both captan and OP compounds, although GST was inhibited only by captan. A screen-printed amperometric multielectrode biosensor was developed for the rapid discrimination of paraoxon and carbofuran in mixtures.[167] Four types of native or recombinant AChEs (electric eel, bovine erythrocytes, rat brain, and *Drosophila melanogaster*) were immobilized by screen printing onto four-electrode thick film sensors in sets each containing AChE. The individual inhibition pattern of each AChE−analyte combination enabled the discrimination of both analytes by the use of neural network data processing.

An enzyme-based monitoring system provides the basis for continuous sampling of OP contamination in air.[168] The system proved suitable for the detection of a range of organophosphates including paraoxon, demeton-S, and malathion. The enzymes BChE and OP hydrolase (OPH) are stabilized by encapsulation in biomimetic silica nanoparticles packed in a column. The resulting immobilized enzyme reactors were integrated using an impinger-based aerosol sampling system for collection of the chemical contaminants in air. The sampling system was operated continuously, and OP detection was performed in real time by single-wavelength analysis of the enzyme hydrolysis products. The sensor system detects OPs based on either enzyme inhibition of BChE or substrate hydrolysis by OPH.

The determination of OPs based on ChE inhibition can be made even more sensitive after being oxidized to oxons. The study by Kim et al. demonstrated that treatment of OP pesticides with excess bromine in acetonitrile is a rapid and efficient method for their oxidation.[169] For the nine OP pesticides tested, all the reactions gave their respective oxons as the single major product with the exception of fenthion, which gave two major products. The inhibitory power of these pesticides on ChE after oxidation was much higher than before oxidation. In another report, the enhanced sensitivity of pH-sensitive AChE biosensors to OPs after oxidation (stronger inhibitor) was also demonstrated.[170]

In addition to common OP and carbamate pesticides or toxins, ChE inhibition can also be used to detect exposure to contaminants such as aflatoxin,[171] glycoalkaloids,[172] fluoride, and heavy metals.[173−176]

Alzheimer's disease is a common form of brain disorder among older people that seriously affects a person's ability to carry out their daily activities. One of the most promising approaches for treating Alzheimer's disease is to enhance acetylcholine levels in the brain using AChE inhibitors such as tacrine, donepezil, rivastigmine, and galanthamine. Hence, the search for new AChE inhibitors is of great interest. Nature is a rich source of biological inhibitors. Using Ellman's colorimetric method, 32 plants used in Thai traditional rejuvenating and neurotonic remedies were collected and the plant methanolic extracts were tested for AChE inhibitory activity.[177] The results showed that methanolic extracts from the roots of Stephania suberosa Forman and Tabernaemontana divaricata (L.) R.Br. ex Roem & Schult at concentrations of 0.1 mg/mL inhibited >90% of AChE activity. At the same concentrations, four extracts from the stems of Piper interruptum Opiz., the seeds of *Piper nigrum* L., the rootbark of Butea superba Roxb, and the roots of Cassia fistula L. showed a 50−65% inhibitory activity on AChE. Another study reports the use of TLC to screen for AChE inhibitors from Amaryllidaceae extracts:[178] the plates were developed and then stained using Ellman's reagent DTNB in order to observe the AChE activity.

An electrophoretically mediated microanalytical method for screening AChE inhibitors in natural extracts has been described.[179] In this method, AChE and a mixture of the substrate and the natural extract were successively injected into the capillary. They were mixed electrophoretically by applying a voltage for a short time. Afterward the voltage was applied to separate the product from the unreacted substrate and the natural extract. The measured peak areas of the product represent the enzyme activity. The inhibitory activity of the natural extract as a whole can be easily assessed by the reduction of the peak area.

Despite the large interest in inhibition-based biosensors, there appears to be some confusion in the literature over what type of responses or quantitative relationships can be expected from immobilized enzyme inhibition biosensors.[180] A variety of linear, nonlinear, logarithmic, and other relationships between inhibition percentage and either inhibition concentration and/or incubation time have all been reported. Zhang et al. presented a general mathematical expression relating the percent of enzyme inhibition to both the inhibitor concentration and the incubation time. The quantitative relationships expected between inhibition percentage and either incubation time and/or inhibitor concentration depend on a variety of physical, chemical, and biochemical parameters associated with the particular design and construction of the biosensing probe.

6. Outlook and Future Perspectives

At present, it has been well-established that ATCh and DTNB are employed for the classic spectrometric assay of ChE. However, the ideal substrate suitable for the electrochemical assay of ChE remains to be established. Various electron mediators and chemically modified electrodes have been employed in order to make thiocholine, the hydrolysized product of ATCh, detectable at lower potential, thus giving a more sensitive signal. It is pertinent, therefore, that alternative substrates or electroactive mediators suitable for the electrochemical assay of ChE with a view to simplifying the analytical procedures be explored.

One challenge for ChE inhibition-based assays for pesticides or other inhibitors is the improvement of assay sensitivity. ChE's from various animal sources or genetically modified enzymes need to be compared. An effective immobilization method improves not only the enzyme activity and stability but also inhibition sensitivity. A biomimetic immobilization of ChE is preferable for this purpose. The use of new materials and nanotechnology has demonstrated numerous advantages over conventional techniques: higher sensitivity, lower detect limits, faster responses, and smaller sizes for both instrument and sample. However, their practical application is still in the early stage, and many challenges remain to be overcome.

The development of organic-phase enzyme assays has attracted considerable interest due to the potential advantages of broadening the analytical field to hydrophobic samples and decreasing the possible interference from hydrophilic species. An organic solvent with an extremely low solubility is preferred since hydrophilic solvents can stripe the enzymes of water essential for enzymatic activity. An oil-in-water emulsion is one of the recommended systems where amphiphilic ChE molecules can be distributed at the interface of two phases and hydrophobic inhibitors are dissolved in the oil drops. The most attractive aspect of this approach is that oil and water are dispersed fully in an emulsion, and thus, the contact probability between enzymes and inhibitors from different phases is increased. In addition, only a little oil is needed for this system, which greatly decreases the influence of the organic solvent on the ChE activity and the use of environmentally toxic and expensive organic solvents.

ChE-based assays give a sum parameter of ChE inhibition, and they are not usually capable of discriminating various toxic compounds in a multicomponent sample. In particular, the simultaneous presence of heavy metals in contaminated samples provides a challenge. However, they remain attractive as an alarm or screening approach because they provide an indication of the total sample contaminations. The savings in both time and cost by using this technique, which is relatively simple and cost-effective, may be enormous.

Current efforts are directed toward the development of more reliable automated and continuous systems for measuring ChE inhibitors under flow conditions.[11] Automation can be achieved by using a computer-controlled programmable valve system, which allows reproducible pumping of the different reagents including buffer solutions, enzymes, substrates, and real samples containing inhibitors.

Although laboratory testing is generally considered to provide more accurate results, field testing is often an effective alternative since it is faster and less expensive with simpler handling procedures amenable to both mass production and instrument miniaturization. Disposable test strips for visual assessment or a portable spectrophotometer containing a test kit are the techniques of choice for field applications. Compact and portable ChE-based devices or biosensors specifically designed for in-field analysis can potentially serve as an alternative to more expensive and complex classical methods.

7. Acknowledgments

We greatly appreciate the support of the National Natural Science Foundation of China (Grants 20975093, 20635020, and 20821063). This work was also supported by the National Basic Research Program of China (Grant 2006CB933201).

8. List of Abbreviations

AChE	acetylcholinesterase
ATCh	acetylthiocholine
NTA-Lys	*N*α,*N*α-bis(carboxymethyl)-L-lysine
BTB	bromothymol blue
BChE	butyrylcholinesterase
BTCh	butyrylthiocholine
CL	chemiluminescence
ChEs	cholinesterases
ChO	cholineoxidase
2,6-DCIP	2,6-dichloroindophenol
DOODA	dioctyloctadecylamine
DAMAB	*N*,*N*-didecylaminomethylbenzene
DTNB	dithiobisnitrobenzoate
DTNA	dithiodinicotinic acid
2-PDS	2,2′-dithiodipyridine
ENFETs	enzyme-modified ISFETs
Ph-F	fluorochrome-tagged physostigmine
GC	gas chromatography
GST	glutathione S-transferases
HPLC	high-performance liquid chromatography
HPLC-MS	high-performance liquid chromatography−mass spectrometry
HPLC/MS/MS	high-performance liquid chromatography/tandem mass spectrometry

ISFET	ion-sensitive field-effect transistor
LB	Langmuir−Blodgett
LSPR	localized surface plasmon resonance
MUA	*N*-mercaptoundecanoic acid
MOSFET	metal-oxide-silicon field-effect transistor
μTAS	micrototal analysis systems
OPH	OPhydrolase
OP	organophosphorus
PCCA	polymerized crystalline colloidal array
2-PAM	pyridine 2-aldoxime methiodide
QDs	quantum dots
QCM	quartz crystal microbalance
SWV	square-wave voltammetry
SAM	self-assembled monolayer
SPR	surface plasmon resonance
TPPS1	tetraphenylporphyrin
TLS	thermal lens spectrometry
TLC	thin-layer chromatography
RSH	thiocholine
TNB	5-thio-2-nitrobenzoic acid
TMB	3,3′,5,5′-tetramethylbenzidine
TMB-4	1,1 V-trimethylenebis-4-formylpyridinium bromide dioxime

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