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Ultra-Sensitive determination of pesticides via cholinesterase-based sensors for environmental analysis.

Frank Davis[a], Karen A. Law[a], Nikos A. Chaniotakis [b], Didier Fournier[c], Tim Gibson[d], Paul Millner[e], Jean-Louis Marty[f], Michelle A. Sheehan[g], Vladimir I. Ogurtsov[g], Graham Johnson[h], John Griffiths[h], Anthony P. F. Turner [a] and Seamus P. J. Higson*,[a]

[a] Cranfield University at Silsoe, Barton Rd, Silsoe, MK45 4DT, UK., Phone: +44 (1525) 863453, Fax: +44 (1525) 863533, Email: s.p.j.higson@cranfield.ac.uk

[b] Department of Chemistry, University of Crete, 71 409 Iraklion Crete GREECE

[c] Institut de Pharmacologie et de Biologie Structurale, Université Paul Sabatier, 205, route de Narbonne, 31077 Toulouse Cedex France

[d] T & D Technology Ltd., Stanley, Wakefield, West Yorkshire, WF3 4AA, UK.

[e] School of Biochemistry and Molecular Biology, University of Leeds, Irene Manton Building, Leeds, LS2 9JT, UK.

[f] Centre de Phytopharmacie, UA CNRS 461, Université de Perpignan, 52 avenue de Villeneuve, 66860 Perpignan Cedex, France

[g] Tyndall National Institute, University College, Lee Maltings, Prospect Row, Cork, Ireland

[h] Uniscan Instruments Ltd, Sigma House, Burlow Road, Buxton, SK17 9JB, UK.

Abstract

This review is focussed towards the development of acetylcholinesterase enzymatic based biosensors for the quantification of trace concentrations of highly toxic pesticides via their inhibitory effect on the enzyme. Initial results were obtained using wild-type enzymes which have a broad spectrum of susceptibility to a variety of pesticides. The sensitivity and selectivity of the enzyme activity was improved by development and screening of a wide range of mutant enzymes. Optimal enzymes were then exploited within a range of sensor formats. A range of immobilisation techniques including adsorption based approaches, binding via proteins and entrapment within conducting polymers were all studied. The incorporation of stabilisers and co-factors were utilised to optimise electrode performance and stability - with both planar and microelectrode geometries being developed. Reproducible quantification of pesticides could be obtained at concentrations down to 10^{-17} M, representing a detection limit hitherto unavailable.

1. Introduction

While pesticides are used extensively within modern agricultural techniques to control insect infestation, increasing concern is being shown towards their indiscriminate use and the long-term effects they may cause to the environment, livestock and human health [1,2]. A significant proportion of the pesticides used within agriculture become washed off or are otherwise lost from the large areas of agricultural land treated surfaces - and for this reason an excess of active ingredient is commonly applied [3]. The problem is compounded by the fact that many pesticides such as DDT have very long lifetimes under environmental conditions. Although organophosphate pesticides (OPs) are now commonly used instead of the organochlorine pesticides due to their lower persistence in the environment whilst still remaining effective, they are, however, neurotoxins and therefore present a serious risk to human health. These compounds may still find their way into our food and water supplies, which necessitates the use of analytical approaches for the reliable detection of pesticides for environmental protection and food safety purposes. Legislation has now been passed to help restrict pesticides within water supplies; European Commission: EU Water Framework Directive 2000/60/EC, European Commission: Drinking Water Directive 98/83/EC, which recommends levels within water supplies of 0.1 mg/l for individual pesticides and 0.5 mg/l for total pesticide. It is likely that with the widespread concerns about these materials that these levels could come down. More recently, the area of biodefense is receiving much attention, with organophosphate based nerve agents also needing to be analysed.

Contemporary methods for environmental determination and/or the monitoring of pesticides include gas and liquid chromatography and various spectroscopic

techniques [4]. Each of these approaches suffers from several disadvantages such as being costly, time consuming, not sufficiently sensitive and/or requiring complex sample preparation [1,5,6]. Continuous monitoring moreover, is not possible with any of these methods and it follows that a simplified analytical approach would prove highly beneficial.

A potential solution to this problem is the utilisation of biosensor technology. Biosensors generically offer simplified reagentless analyses for a range of biomedical and industrial applications and for this reason thie area has continued to develop into an ever expanding and multidisciplinary field during the last couple of decades. Electrochemical techniques are amongst the easiest and most inexpensive methods for detection of binding events and many groups have previously demonstrated the fabrication of enzymatic and affinity based sensors that lend themselves to interrogation by either (i.) amperometric or (ii.) impedimetric approaches.

Much of the work described was carried out within a collaborative project between a number of academic and industrial groups under the remit of the SAFEGARD consortium, an EU funded Framework 5 research contract ref QLRT-1999-30481. The various expertises available from the academic and industrial collaborators made this project feasible.

The detection of many pesticides at extremely low levels can be best achieved not by direct detection of the pesticide itself but rather by detection of its inhibitory effects on enzyme reactions. An enzyme-electrode is first constructed and its response when exposed to a suitable concentration of its substrate determined. When an electrode is

then exposed to a dilute pesticide solution, the pesticide interacts with the enzyme and diminishes (or completely destroys) its activity. This inhibition can then be easily quantified by further exposure to the initial substrate concentration and comparison with the response prior to pesticide exposure.

The detection of organophosphate and other pesticides based on the inhibition of the enzyme acetylcholinesterase by these compounds has received considerable attention primarily due to high specificity and sensitivity [1,7-16]. Cholinesterases, such as acetylcholinesterase catalyse the hydrolysis of choline esters to the corresponding carboxylic acid and choline; Eqn. 1.

Eqn. 1.

$$\text{Acetylcholine} + H_2O \xrightarrow{\text{acetylcholinesterase}} \text{choline} + \text{acetic acid}$$

The use of electrochemical techniques combined with biological molecules has been extensively reviewed [17] and will not be discussed in detail here. The most widely used method for the AChE containing electrodes is via the simple amperometric detection of the product of the ester hydrolysis enzyme catalysed reaction [17].

A typical approach is to utilise a substrate which when hydrolysed by the enzyme gives rise to a product which can be easily detected electrochemically. Thiocholine can be easily detected using screen-printed carbon electrodes doped with cobalt phthalocyanine (CoPC) [18,19], which acts as an electrocatalyst for the oxidation of thiocholine at a lowered working potential of approximately +100mV (versus

Ag/AgCl) [18,19], thereby minimising interference from other electroactive compounds; Eqn. 2.

Eqn. 2.

acetylthiocholine chloride + H_2O ----------> thiocholine(red) + acetic acid + Cl^-

2 thiocholine(red) ---------> thiocholine(ox) + 2e− + 2H+ (at 100 mV vs. Ag/AgCl)

A similar approach utilises *p*-aminophenyl acetate [20].

There are problems with this approach since enzymes isolated from natural sources such as the electric organ of electric eels often display low sensitivity and selectivity to the wide range of potential pesticide targets [21]. A possible solution to this is the development of a multisensor array where a variety of genetically modified acetylcholinesterases are immobilised on an array of electrochemical sensors and the responses from these are then processed via a neural network.

A wide variety of methods exist for the immobilisation of enzymes on a sensor surface. Screen-printed carbon electrodes are often the favourite base for these sensors due to their inexpensiveness and ease of mass-production. Methods used for the construction of AChE containing electrodes include: simple adsorption from solution [22], entrapment within a photo-crosslinkable polymer [20,24], adsorption from solution onto microporous carbon and incorporation into a hydroxyethyl cellulose membrane [25], binding to a carbon electrode via Concanavalin A affinity [26,27] and entrapment within conducting electrodeposited polymers [28].

2. Application

2.1 Synthesis of the acetylcholinesterase.

Earlier work in this field [29] indicated that acetylcholinesterase enzymes would be suitable biomolecules for the purpose of pesticide detection, however, it was found that the sensitivity of the method varied with the type and source of cholinesterase used. Therefore the initial thrust of this work was the development of a range of enzymes via selective mutations of the *Drosophila melanogaster* acetylcholinesterase (*Dm.* AChe). For example mutations of the (*Dm.* AChe) were made by site-directed mutagenesis expressed within baculovirus [30]. The acetylcholinesterases were then purified by affinity chromatography [31]. Different strategies were used to obtain these mutants, namely: (i.) substitution of amino acids at positions found mutated in AChE from insects resistant to insecticide, (ii.) mutations of amino acids at positions suggested by 3-D structural analysis of the active site, (iii.) Ala-scan analysis of amino acids lining the active site gorge, (iv.) mutagenesis at positions detected as important for sensitivity in the Ala-scan analysis, and (v.) combination of mutations which independently enhance sensitivity. The activity of the enzymes was determined photometrically at 412 nm using the Ellman method [32]. The use of these different strategies allowed the development of sensitive enzymes, for a mutant which was 300-fold more sensitive to dichlorvos than the Drosophila wild-type enzyme - and 288,000-fold more sensitive than the electric eel enzyme which is commonly used to detect organophosphates. The most effective method of increasing the sensitivity to the pesticide appeared to be via the incorporation of hydrophobic amino acids at the rim of the active site of the enzyme [25,33]. For example, in solution the sensitivity to methamidophos of a genetically modified *Drosophila* AChE was one order of magnitude higher than a commercial electric eel AChE [33]. When immobilised in a

biosensor, this led to a useful working analytical range for the *Drosophila* AChE electrode of 0.5-100 ppb methamidophos as against 0.05-24 ppm for the commercial AChE electrode.

2.2 Immobilisation of the enzymes.

Several different approaches have been developed within the group of the SAFEGARD consortium to immobilise the engineered AChEs. The simplest of these was reported by Bonnet *et* al [22] in which a screen-printed graphite electrode was exposed to a solution of commercial electric eel AChE in phosphate buffer. The resultant enzyme electrodes were then used to detect acetylthiocholine chloride (10^{-3} M) which gave currents in the range of 225 nA. Inhibition studies with chlorpyrifos ethyl oxon (exposure time 10 min) were performed and gave a detection limit of 1.2 ng l^{-1} with good operational stability. The absence of diffusion barriers, however, gave a high level of sensitivity, although there was high variability in response between electrodes. In the work of Andreescu *et al* [23], a comparative study where the enzyme was immobilised using the following techniques was made. This included;

(a) A mixing of AChE with graphite, tetracyanoquinodimethanide (TCNQ, used as a mediator), hydroxyethylcellulose (HEC) and a methyltrimethoxysilane based sol-gel which was then deposited on a screen-printed working electrode surface as a paste. This was then allowed to dry.

(b) Screen-printing a graphite/TCNQ/HEC composite electrode, then printing a layer consisting of the enzyme and a 30% solution of a photopolymerisable poly(vinyl alcohol)/styryl pyridinium copolymer on top of the electrode and finally irradiation with UV light to photocrosslink the polymer.

(c) Screen-printing a graphite/TCNQ/HEC composite electrode and then printing a layer containing a nickel compound attached to a silica support. This was then exposed to a solution of a histidine$_6$-tagged AChE in phosphate buffer, with the histidine tag binding strongly to the immobilised nickel compound.

The three types of electrode were exposed to solutions of acetylthiocholine chloride and the resulting current recorded. These gave slightly different calibration curves over the concentration range 0-2.5 x 10^{-3} M of substrate, with the nickel containing composite being the least sensitive. The nickel composite also gave the poorest storage performance, with the other two electrodes being stable up to 12 days, and the poorest reproducibility also being observed for the nickel binding method.

Inhibition studies were made with chlorpyrifos ethyl oxon with the sol-gel method giving the largest linear range (0- 6 x 10^{-8} M), although the nickel-binding method gave an electrode which was more sensitive at lower concentrations. The sol-gel electrode also gave good response behaviour to paraoxon and dichlorvos.

Further work [34] also compared enzyme electrodes formulated using the photocrosslinking technique above - with the electrodes being treated by simple immobilisation of AChE inside a matrix of bovine albumin crosslinked by glutaraldehyde. A variety of experimental conditions were utilised. The glutaraldehyde crosslinking technique has the advantage of simplicity and gives electrodes which have fast response times while being robust and reproducible. They did require a far higher enzyme content (80 mUA), however, to give similar responses

to those of the photopolymerised electrodes containing 0.7-1.0 mUA of enzyme. It should also not be forgotten that this greatly increases the expense of such systems.

Microporous carbon was also studied as a potential substrate for binding of AChE [25,35]. Discs cut from a commercial porous carbon rod were cleaned and then exposed to a solution of AChE in phosphate buffer for 20 hours to allow for simple physisorption and chemisorption of the enzyme. Initial tests using electric eel AChE [35] gave linear detection of dichlorvos in the range 10^{-6}-10^{-12} M. The sensitivity of this method was increased still further by utilisation of the genetically engineered AChE mentioned earlier, with the detection limit of these systems being extended down to 10^{-17} M [35].

Instability of the mutant AChE can be a problem with up to 50% of its activity in solution being lost in 10 days. This led to a study in which the enzyme was immobilised in porous silica (pore size 10 nm) or porous carbon (<70 nm) beads [36]. The AChE is known to be approximately 6 nm in size and therefore it is thought that entrapment within the pores could well inhibit unfolding of the enzyme, so enhancing its stability.

Following immobilisation, the beads were dispersed in a aqueous solution of HEC and cast onto Pt electrodes. Activity tests showed that leaching of immobilised enzyme was 2.5 times slower than that of free enzyme dispersed in HEC. Comparisons of activity to acetylthiocholine after 72h constant operation showed a large stability enhancement for enzymes immobilised on both silica and carbon when compared to dispersion in HEC [36].

Affinity binding was also used to bind AChE to a working electrode surface [26]. Amino-grafted silica beads were used as the starting point and reacted with glutaraldhyde. The resultant beads containing active aldehyde groups on the surface were then treated with Concanavalin A, a lectin type protein with binding affinity for mannose, a sugar which is present at the surface of AChE. Finally the protein-grafted silica beads were treated with a solution of commercial electric eel AChE [26]. Treatment with divinyl sulfone followed by a disaccharide was used as alternative activating step before Concanavalin A adsorption. Monitoring of enzymatic activity showed binding of the AChE only for systems containing sugar/Concanavalin A affinity links, indicating that unspecific adsorption did not lead to immobilisation of the enzyme. The beads were then mixed with graphite/TCNQ composite and cast onto a screen-printed working electrode.

Amperometric activity of the electrodes in thiocholine before and following exposure to solutions of pesticides was measured. Sample to sample reproducibility was found to be favourable (RSD 6.6%), as was stability with electrodes being shown to be capable of being stored for up to two months at -18^{o}C. Linear detection of chlorpyrifos methyl oxon by inhibition was obtained between 1 x 10^{-8}-5 x 10^{-8} M by this approach.

A similar method was used [27] to directly immobilise AChE on the electrode. A screen-printed carbon electrode was treated with a Nafion/heptylamine mixture. The amino groups were then activated with divinyl sulfone and then treated with a disaccharide. This was then used to bind first Concanavalin A and then electric eel AChE via affinity binding. The resultant electrodes had similar reproducibility to the

silica-containing analogues with no enzyme leakage occurring upon storage for 2 hours in buffer. Bovine albumin was used in this instance to block non-specific binding. The electrode activity was completely inhibited upon exposure to chlorpyrifos methyl oxon (10^{-5} M), but could be completely regenerated simply by exposing the electrode to fresh AChE solution; this behaviour was observed for three inhibition/regeneration cycles.

2.3 Use of microelectrodes.

Another potential method for immobilising AChE is to entrap the enzyme within a conducting polymer such as polyaniline. The entrapment of biological molecules within conducting polymers has been widely studied and extensively reviewed elsewhere [37]. All the methods described so far in this paper have been related to the production of planar electrodes. Microelectrodes offer several advantages over conventional larger working electrodes within biosensors, since they experience hemispherical solute diffusional profiles, and it is this phenomenon that can impart stir independence to sensor responses whilst also offering lowered limits of detection.

Individual microelectrodes offer very small responses and one approach for overcoming this problem is to use many microelectrodes together in the form of an array to allow a cumulative and so larger response to be measured. Microelectrode arrays may be fabricated by a number of approaches although techniques such as photolithography or laser ablation have to date proved cost prohibitive for the mass production of disposable sensor strips. We have previously described a novel sonochemical fabrication approach [38, 39] for the production of microelectrodes, that lends itself to the mass production of sensor arrays.

The method of producing microelectrodes will be described in more detail within the protocol (protocol 25). The method is as shown schematically in Fig. 1. A conducting surface, for example formed by screen printed-carbon can be insulated by deposition of poly(*o*-phenylene diamine). Sonochemical ablation has been shown to form pores in this insulating surface [39] with population densities of up to 2 x 10^5 pores cm^{-1}. Electrochemical deposition of conducting polyaniline at these pores can be performed and used to grow protrusions of polyaniline at the surface [38] and if AChE is included in the deposition solution, the enzyme may be entrapped within a conducting polyaniline matrix [28]. These arrays of polyaniline protrusions can be visualised by scanning electron microscopy [40] (Fig. 2) and display the typical stir-independent behaviour of microelectrodes [28].

In this way a sonochemically-fabricated microelectrode array was used to form an array of conducting microelectrodes [28] containing a genetically modified AChE which had been modified to maximise pesticide sensitivity. Use of a I^{125} labelled AChE meant that the amount of enzyme deposited could be measured and in this instance corresponded to 0.15 units activity. Measuring the amperometric response of the electrode in acetylthiocholine before and following exposure to paraoxon solutions allowed a measurement of the inhibition of enzyme activity. Levels as low as 10^{-17} M paraoxon could be reproducibly detected [28]. Although very low, these levels are comparable to those determined using acetylcholineesterase immobilised on microporous conductive carbon [25].

2.4 Multiple pesticide detection.

One major problem with determining pesticides in real samples is that one or several of a range of pesticides could be present. Therefore we need a sensor that can interrogate the sample and determine not only which pesticides are present but at what levels. One possible method is to manufacture a sensor, usually by screen-printing, containing multiple working electrodes with each containing a different AChE. Pattern recognition software can then be used to monitor the varying inhibitory response pesticides and mixtures of pesticides. Alternatively a range of single AChE electrodes can be manufactured and then incorporated into a flow injection system so that they are all simultaneously exposed to the sample, with responses being monitored and pattern recognition software used as before.

A series of multielectrode sensors were developed based on *Drosophila* mutant AChE immobilised via photocrosslinking onto screen-printed carbon electrodes [8]. Four different mutant and wild type AChE were evaluated for their sensitivity to the organophosphate paraoxon and the carbamate pesticide carbofuran. The response of the electrodes in thiocholine before and following a fifteen minute exposure to solutions of the pesticides was compared. The data was then processed using a feed-forward neural network generated with NEMO 1.15.02 as previously described [8, 9]. Networks with the smallest errors were selected and further refined. This approach together with varying the AChE led to the construction of a sensor with capability to analyse the binary pesticide mixtures.

When solutions of individual pesticides were used, concentrations as low as 0.5 μg/l (10^{-9}M) could be determined. When binary mixtures with pesticide levels from 0-5 μg/l were measured, the concentration of each pesticide could be determined within

the range with errors of 0.4 μg/l for paraoxon and 0.5 μg/l for carbofuran. Similar levels were obtained when river water samples spiked with pesticide were used but with a higher degree of inaccuracy. When different mutant AChE's were utilised, binary mixtures of the very similar pesticides paraoxon and malaoxon could be analysed in the range 0-5 μg/l, with resolution of the two components with accuracies of the order of 1 μg/l. The use of more sensitive and selective mutant enzymes together with the addition of extraction and concentration steps to the assay could greatly enhance the methods range and accuracy.

A flow injection system combined with an enzyme electrode was used to detect and quantify a variety of pesticides [41]. Photocrosslinkable poly(vinyl alcohol) was used to immobilise AChE onto platinum wire working electrodes. These were then placed inside a flow injection cell and the electrochemical response to injections of thiocholine measured. A series of measurements were made before and following the injection of a pesticide solution. Under constant flow, the sensors were found to be stable for several days. The inhibition of the current after exposure to various pesticide solutions was measured with detection limits using mutant AChEs being found to be as low 1.1 μg/l (Chlorpyrifos oxon), 30 μg/l (paraoxon) and 25 μg/l (malaoxon). What makes this system of interest is that it could potentially be used for multiple tests with the sample electrode, since injection of and incubation of the electrode with pyridine-2-aldoxime methochloride reversed the inhibition effect of the pesticide. Detection of pesticides in spiked river water samples was also achieved.

Some of the work described previously showed diminution of the biosensor performance when pesticide solutions using river water rather than laboratory water

were used. This is thought to be partially due to other compounds present within river water affecting electrode performance. A system containing triple enzyme electrodes within a flow injection system was developed in an attempt to combat this [23]. Three different AChE variants were immobilised on screen-printed electrodes by photocrosslinking, one a wild type *Drosophila*, the second a mutant with extremely high sensitivity to pesticides and the third a wild type electric eel AChE which is relatively resistant to pesticide. However any matrix interference would affect both electrodes equally and therefore can be subtracted, allowing us to distinguish inhibition due to the presence of non-pesticide inhibitors, e.g. Hg from specific interactions which occur only if pesticides are present. Limits of detection for the pesticide omethoate were found to be 2×10^{-6} M for the wild type *Drosophila* and 10^{-7} M for the mutant - levels which caused only minimal inhibition of the electric eel AChE control.

Heavy metals and hypochlorite can both inhibit AChE [23] and so similar tests for pesticides were repeated in solutions containing either 20 mg/l Hg^{2+} or 0.1 mg/l $NaClO_4$. In both cases large inhibition effects were noted for both the enzyme electrodes, not just the mutant, so indicating the presence of a non-specific interferent. When river water was introduced to the system, no inhibition effects were observed, however, when omethoate spiked river water samples were used, then inhibition effects could be measured for the mutant with similar levels of sensitivity to when pure water was used as the matrix.

As an alternative to simple AChE electrodes, a bienzyme system containing AChE and tyrosinase which utilised phenyl acetate as a substrate has been developed [42].

The AChE hydrolyses the phenyl acetate to phenol which the tyrosinase enzyme oxidises to *p*-quinone which can in turn be detected electrochemically. The bienzyme system was found to be less sensitive than the AChE electrode, although it did display a large tolerance for hexane.

Micro-electrode arrays containing AChE were also utilised within a flow injection system [40]. A system was developed where a sample was separated and flushed simultaneously through eight cells, each containing a screen-printed electrode and fitted with a separate bespoke mini-potentiostat (Fig 3). This allowed multiple measurements to be made on a single water sample using multiple electrodes, each specific for a different pesticide due to inclusions of different AChE mutants in each of the electrodes. Pattern recognition software could then be utilised to deduce the pesticide levels in a potentially complex sample.

Early results indicate a high sensitivity for pesticide detection, with the system being capable of detecting dichlorvos at concentrations as low as 1×10^{-17} M and parathion and azinphos both at concentrations as low as 1×10^{-16} M [40].

2.5 Signal processing for pesticide detection.

The development of user-friendly automated instrumentation able for identification and quantitative detection of pesticides is needed for a wide variety of application areas. For the identification and quantification of the pesticide type the multi sensor approach combined with pattern recognition software is highly promising. To enable pesticide quantification, special algorithms for the signal processing of the biosensor response have been developed.

These algorithms can be divided into two groups. The first group consists of algorithms related to the signal processing of a separate sensor response on a pesticide injection. They decrease the influence of noise on the measurements by increasing the signal-to-noise ratio thereby providing a lowering of detection limit and increasing the sensitivity and reproducibility of the instrumentation.

The second group consists of algorithms associated with the pesticide concentration quantification. In this case, the initial data is the processed sensor response for an unknown pesticide concentration and the parameters of the calibration curve (which is derived from preliminary experimental calibration measurements for a range of standard pesticide concentrations) or alternatively, a set of sensor responses obtained by addition of known amounts of pesticide to the analysed sample. This group of algorithms allows the automation of the pesticide quantification, thereby enabling the use of the instrumentation by unskilled personal. This removes the sensing platform from specialized laboratories to the realm of the end-users.

The structure of the algorithm for the developed software is presented in Fig.4. It integrates biosensor signal processing together with pesticide quantification algorithms and includes:

- Preliminary biosensor signal processing
- Analytical signal extraction, and,
- Analytical signal processing.

Preliminary biosensor signal processing combines the analysis of the background signal and biosensor response and smoothing/filtration of the biosensor response upon pesticide injection. Its purpose is to increase the signal-to-noise ratio of the biosensor response resulting from pesticide injection by using an optimal smoothing/filtration procedures, the parameters of which are defined by the analysis of background signal and the biosensor response after pesticide injection in the time and frequency domains. Application of this approach to the electric eel AChE electrode demonstrated that the biosensor background signal in the time domain represents Gaussian noise with non-zero medium. The correlation time was equal to 17.47 s which defined the lower limit of the integration time for noise filtration. In the frequency domain the background signal presented mainly uniform distributed noise with a small region below 8 mHz with flicker noise type of frequency dependence (1/f function).

Analysis of biosensor response on the pesticide injections with different acteylthiocholine concentrations in the range of 1 – 50 mM displayed a shape of normalised sensor response that only slightly depended on the pesticide concentration. Signal time (the time interval containing 90% of signal energy), which gives the upper limit of the integration time for the signal filtration, decreased only by 16% from 100s if the concentration increased by two orders of magnitude. In the frequency domain the biosensor responses presented a bell-shaped profile where the frequency band of the signal (the band containing 90% of the signal energy) slightly increased with increasing pesticide concentration. The filter band equal to 5 mHz can be selected as a lower limit for the frequency filtration approach.

Examination of the different algorithms for filtration/smoothing of the biosensor response, which included low frequency filtration, Gaussian kernel and running median smoothing, demonstrated that the running median smoothing method could be recommended. This is due to its good adaptability to fast signal variations, which are typical for the biosensor response on a pesticide injection.

The analytical signal extraction block is based on determination of the maximum signal response and includes the following sections: selection of response extremes, determination of the extreme parameters, elimination of the weak extremes and calculation of the analytical signal for pesticide quantification. The differences between the maxima in the biosensor response and baselines were used for the calculation of analytical signals, where the line drawn between two nearby minima within the limits of each injection was taken to be the base line.

This analytical signal of the biosensor was used for:

- calculation of the biosensor calibration parameters (slope and intercept of the calibration line) by statistical processing of the biosensor responses following pesticide injections with known concentrations, or,
- pesticide quantification in the sample by means of calibration parameters or, in case of need more accurate data, by means of the standard addition software analogous to the algorithm described in [43]

3. Conclusions.

This chapter describes the wide range of research undertaken by several groups during the course of the SAFEGARD European Commission funded Framework V project. A wide range of mutant acetylcholinesterase enzymes have been obtained with some being determined to have sensitivities to selected pesticides orders of magnitude greater than wild type enzymes. A wide range of immobilisation techniques have been studied to develop sensitive and selective enzyme electrodes which can measure concentrations of a range of pesticides down to levels hitherto undetectable (1×10^{-17} M).

Other techniques such as use of multiple electrodes, pattern recognition software and flow injection techniques have enabled the subtraction of matrix effects such as heavy metals from the system as well as the determination of pesticides in systems containing more than one compound. The signal processing algorithms allow automation of the pesticide quantification enabling use of the instrumentation by unskilled personal, thereby removing this sensing platform from specialized laboratories and making it available to the end-users. Thus this application could conceivably be utilised in the field as well as under laboratory conditions. The relative low cost of electrochemical technology compared with many of the other technologies used makes it an attractive alternative, especially if the enzyme electrodes can be inexpensively mass-produced using screen-printing to allow single shot use.

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Fig 1. Schematic of sonochemical microelectrode formation: (a). formation of the insulating layer on the electrode surface, (b). sonochemical ablation leading to formation of microelectrode pores, (c). electropolymerisation of aniline and AChE at the pores to form enzyme microelectrodes.

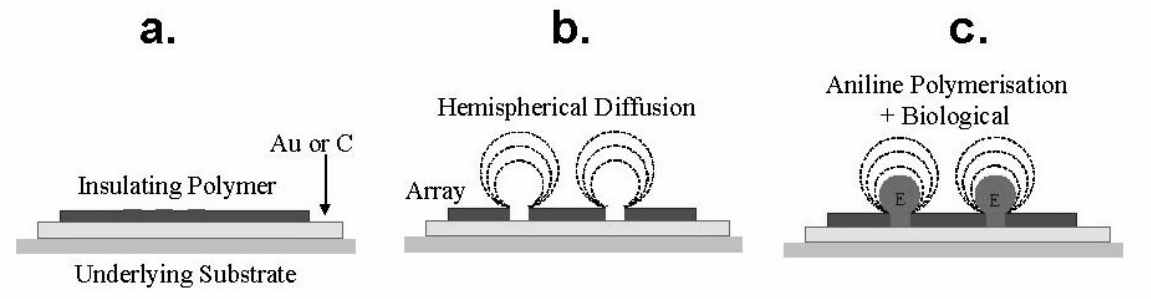
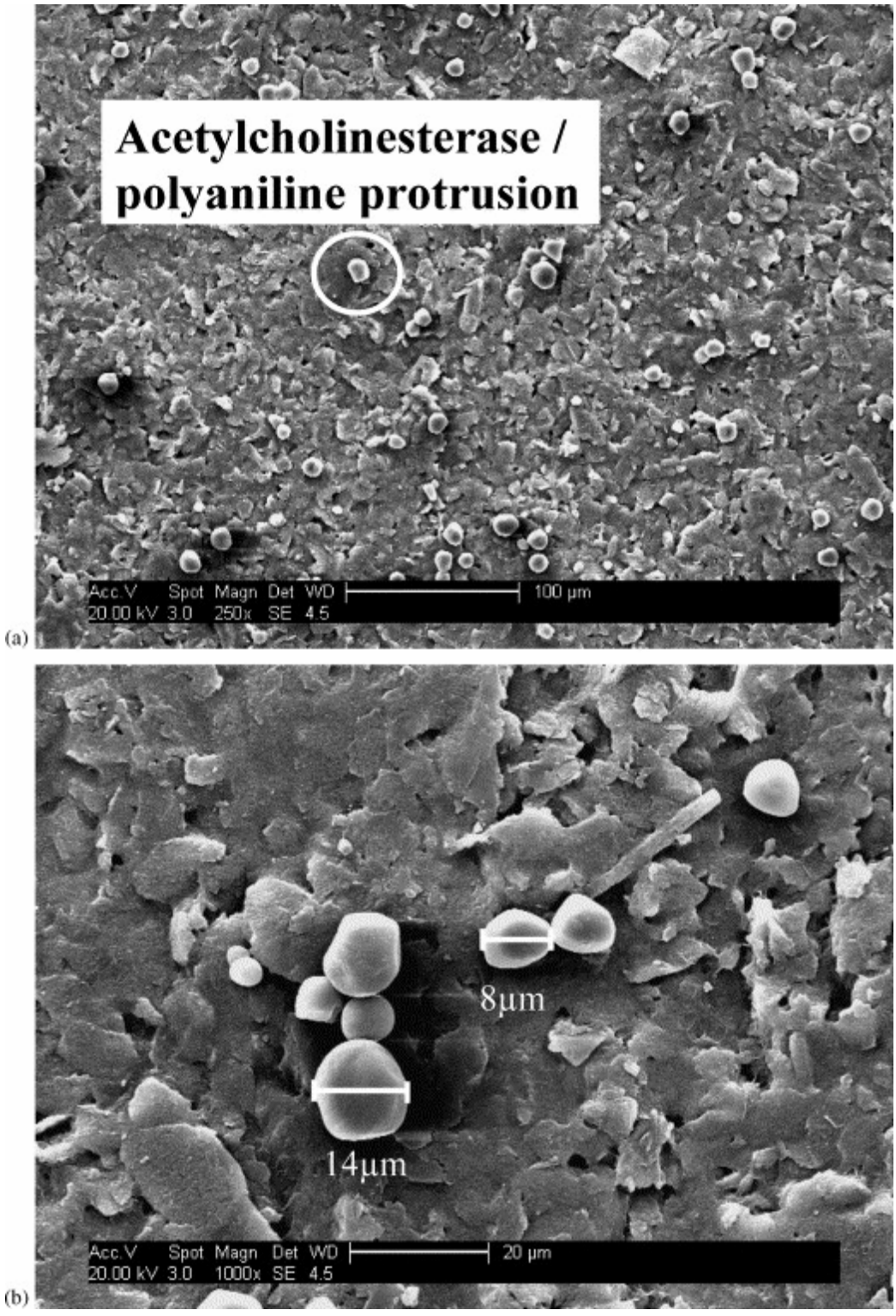
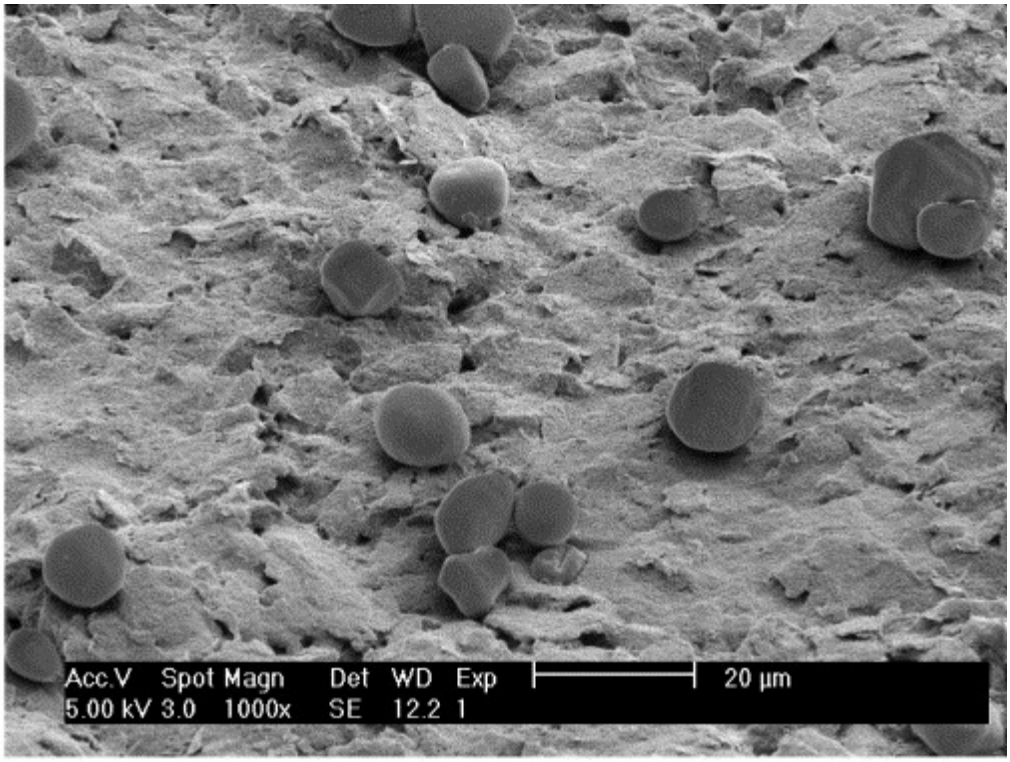
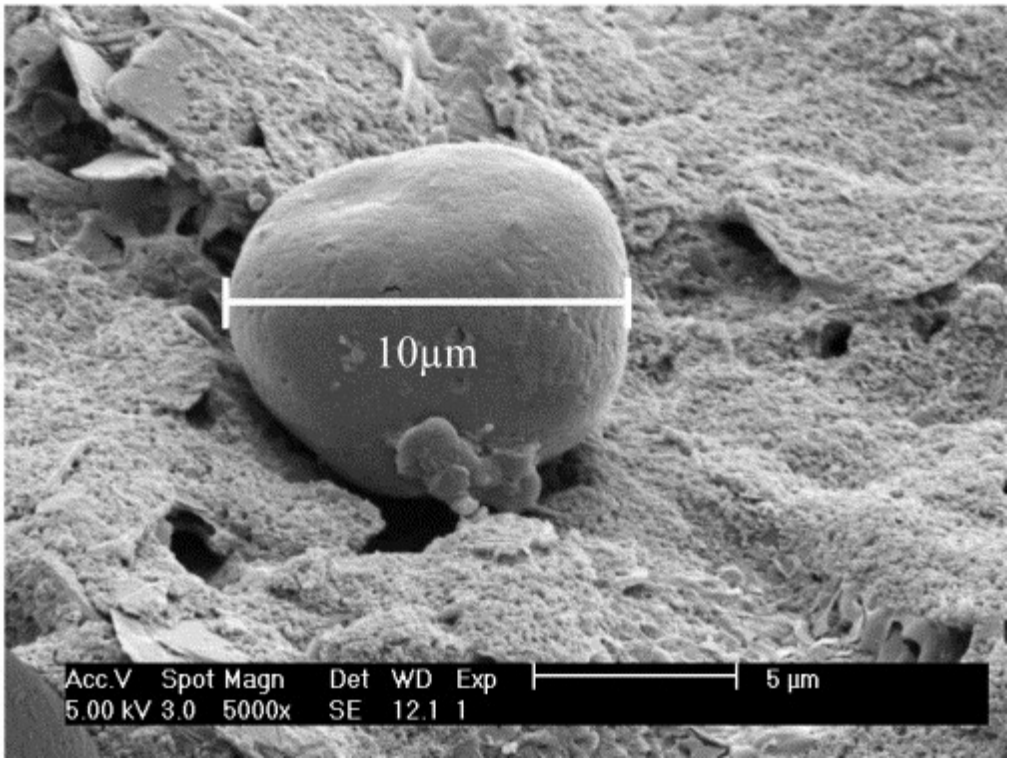


Fig. 2. Scanning electron micrographs of enzyme containing polyaniline protrusions at (a) 250×, (b) 1000×, (c) side view at an angle of 50^{o} 1000×, (d) side view 5000×.




Acc.V Spot Magn Det WD Exp
5.00 kV 3.0 1000x SE 12.2 1
20 µm

(c)


10µm
Acc.V Spot Magn Det WD Exp
5.00 kV 3.0 5000x SE 12.1 1
5 µm

(d)

Fig. 3. Flow-injection analyser. (A) Pump set at 1 ml min^{-1}, (B) injection valves for substrate and pesticide samples, (C) one of eight potentiostats, and (D) flow cell which comprises one sensor.

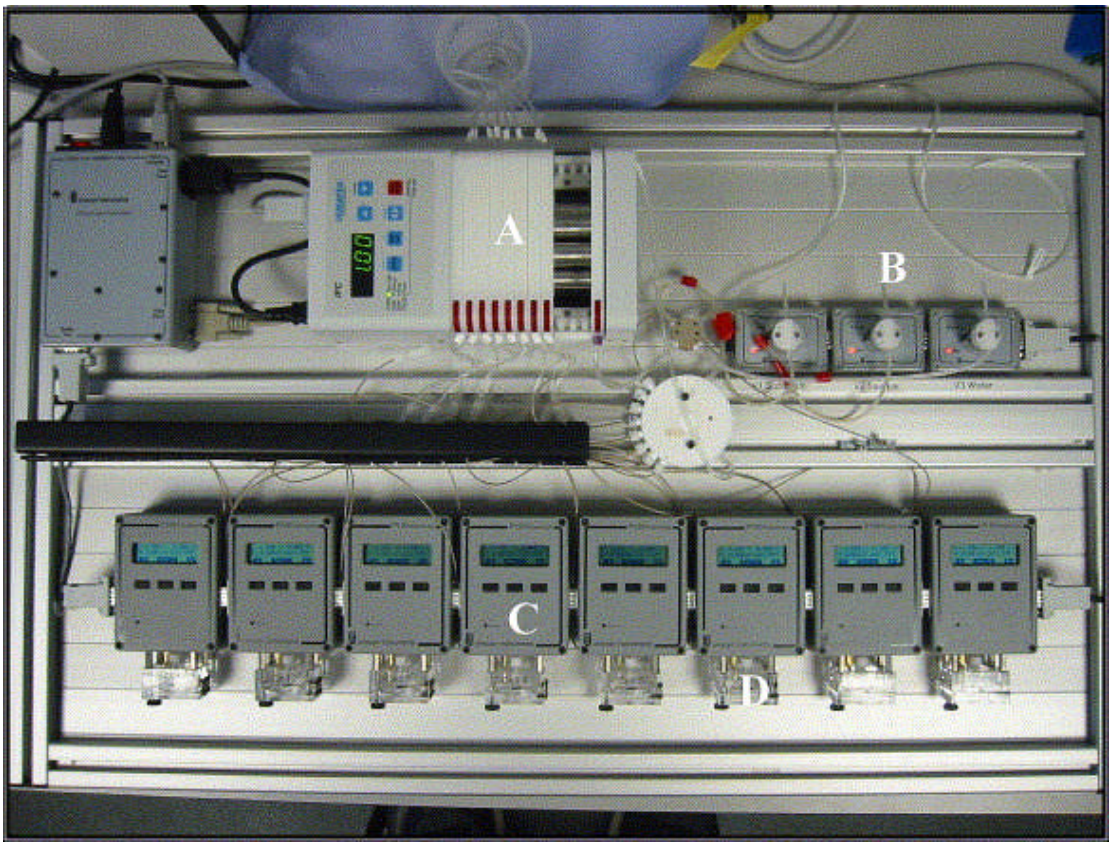


Fig 4. Flow-chart of software for pesticide quantification.