Optical Biosensor with Multienzyme System Immobilized onto Hybrid Membrane for Pesticides Determination

Lyubov Yotova[1*], Nourelhoda Medhat[1,2]

[1]*Department of Biotechnology*
University of Chemical Technology and Metallurgy
8 Kl. Ohridski Blv., 1756, Sofia, Bulgaria

[2]*Biomedical Engineering Department, Faculty of Engineering*
Minia University, Minia, Egypt
E-mails: LYotova@tea.bg, bio_eng_nour@yahoo.com

[*]*Corresponding author*

Received: November 21, 2011 **Accepted: December 27, 2011**

Published: January 31, 2012

Abstract: *A construction of optical biosensor based on simultaneous immobilization of acetylcholinesterase and choline oxidase enzymes for the detection of pesticides residues is described. Different kinds of novel SiO_2 hybrid membranes were synthesized to be suitable for optical biosensors using sol-gel techniques. The bioactive component of the sensor consists of a multi-enzyme system including acetylcholinesterase and choline oxidase covalently immobilized on new hybrid membranes. The sensor exhibited a linear response to acetylcholine in a concentration range of 2.5 - 30 mM. Inhibition plots obtained from testing carbamate (carbofuran) pesticides exhibited concentration dependent behaviour and showed linear profiles in concentration ranges between 5×10^{-8} - 5×10^{-7} M for carbofuran. The factors affecting the constructed optical biosensors were investigated.*

Keywords: *Optical biosensors, Pesticides, Choline oxidase, Acetylcholinesterase.*

Introduction

Recently, many types of biosensors have been developed and used in a wide variety of analytical settings including biomedical; health care; drug design; environmental monitoring; detection of biological, chemical, and toxic agents; biotechnology; physics; research and others. Several enzyme sensors have been developed using electrochemical [10, 12, 17, 20], acoustic [8, 9, 21] or optical biosensors [2, 6, 16, 19, 23].

A pesticide is any substance or mixture of substance intended for preventing, destroying, repelling or mitigating any pest [5]. Pesticides are designed to kill or repel pests but may be harmful to other organisms, including humans. Pesticides contribute significantly to overall cancer mortality. They have the potential to cause adverse effects to the nervous system in humans at low concentrations and can be very ecotoxic to aquatic organisms, birds and bees [7]. The current pesticides include a wide diversity of chemical types, carbamates and organophosphates being two major classes. Both classes of pesticides act as AChE inhibitors.

In the past, the widely used methods for determination of pesticides are liquid/gas chromatography and immunoassays. These methods are sensitive and allow discrimination among different pesticides compounds but require tedious sample pretreatment and sophisticated instruments. Therefore, it is essential to develop a rapid and sensitive screening method to ascertain the presence of these compounds [14].

Accordingly, a lot of effort has been devoted to developing relatively inexpensive enzymatic methods for the industrial processes and environmental monitoring. Biosensors represent an alternative method for quick detection of neurotoxins and have been an active research area for several years. The majority of these biosensors are based on the inhibition reaction of the enzymes acetylcholinesterase (AChE) or butyrylcholinesterase (BuChE) [1, 2, 4-7, 10, 14-17, 19, 20, 23].

Detection of pesticides using fiber optic biosensors based on oxygen measurements are more versatile than electrochemical biosensors for their flexibility in design and signal transduction. Optical sensors have advantages in terms of response time, costs, and size. Their advantages include virtually zero oxygen consumption by the sensor, excellent performance at low pO_2 levels, and the ability to measure gaseous as well as dissolved oxygen. Optical sensors don't require a reference signal such as is necessary in all potentiometric sensors where the difference between two absolute potentials is measured. Optical fibers have various lengths and diameters and can be adapted for either single- or multianalyte detection. Because light is transmitted over long distances in the fiber, a detector can be located at some distance from the sensor tip, allowing the fiber to be used for remote or hazardous applications. In addition, fibers can be miniaturized, which is suitable for in vivo applications [13].

The purpose of the present study is construction of a fiber optic biosensor based on simultaneous covalent immobilization of acetylcholinesterase and choline oxidase enzymes on novel hybrid membranes for the detection of pesticides' residues. Immobilization of AChE on various materials has been reported by many researchers. However, AChE immobilization on membranes similar to ours has not been investigated before. The factors affecting the constructed optical biosensor were investigated. The influence of the selected membrane on the inhibition constant (K_i) of carbofuran will be demonstrated.

Materials and methods

Acetylcholinesterase (EC 3.1.1.7, Type VI-S from electric eel, AChE was supplied by Sigma, Germany); Choline oxidase (ChO) (E.C. 1.1.3.17, from Alcaligenes sp. was supplied from Sigma, Germany); acetylcholine chloride (AChCl, from Fluka, Switzerland); Carbofuran was acquired from Riedel-de Haën.

Construction of the sensor

Four types of novel hybrid membranes were synthesized in our previous work [11]. These membranes were Ethyltrimethoxysilane [ETMS] with Cellulose acetate propionate high molecular weight [CAPH] and copolymer between acrylamide and acrylonitrile [AA/AN]; Methyltriethoxysilane [MTES] with Cellulose acetate propionate high molecular weight [CAPH] and copolymer between acrylamide and acrylonitrile [AA/AN]; Ethyltrimethoxysilane [ETMS] with Cellulose acetate propionate low molecular weight [CAPL] and copolymer between acrylamide and acrylonitrile [AA/AN]; and Methyltriethoxysilane [MTES] with Cellulose acetate propionate low molecular weight [CAPL] and copolymer between acrylamide and acrylonitrile [AA/AN]. The covalent immobilizations of AChE and ChO enzymes were carried out simultaneously on the hybrid membranes in our previous work where the catalytic properties of the enzymes were investigated [24].

The optical biosensor was constructed using the membrane with the highest relative activity for simultaneously immobilized AChE and ChO enzymes. The sensor was based on two sequential enzymatic reactions. The enzyme AChE catalyzed the hydrolysis of acetylcholine into choline and acetic acid. Then enzyme ChO catalyzed the oxidation of choline to betaine and hydrogen peroxide, oxygen was used as a co-substrate. These enzymatic reactions were coupled with the optical determination of oxygen consumption.

The measurements were done using the spectrophotometer and the oxygen probe of Avantes Spec 2048 (Avanes Co. Inc). Oxygen probe is based on the quenching effect of the oxygen on the fluorescence of the ruthenium complex. The AvaLight-LED-475 light source used to emit light with a wavelength of 475 nm. Measurements of the fluorescence are performed at 600 nm. A high signal corresponds with low oxygen level and vice versa.

Using oxygen probe of Avantes fiber optic device, the membrane with immobilized enzymes acetylcholiesterase and cholin oxidase was fixed to the surface of the oxygen electrode by a dialysis membrane. For each measurement, the biosensor was dipped into the measuring cell containing 7.5 mM of Tris buffer, pH 8.0 at 25°C. The initial acetylcholinesterase activity was measured by injecting samples of the substrate solutions (100 mM acetylcholine prepared in the Tris buffer). After that the solution of pesticide (carbofuran) was injected in the measuring cell. The consumption rate of oxygen was used as an indicator for the inhibited enzyme activity. The electrode was washed up with distillated water. The whole measurement cycle was repeated for other pesticide solutions. The inhibition percentage at each measurement was calculated using the following equation:

$$\text{Inhibition } (\%) = [(E_0 - E)/E_0] \times 100, \quad (1)$$

where E_0 is the initial inhibited sensor activity and E is the inhibited sensor activity. The sensitivity of the biosensor toward acetylcholine was measured.

Using spectrophotometer of Avantes fiber optic device, the absorbance measurements were done in cuvette holder for the determination of AChE's activity at wavelength 412 nm in the presence and absence of carbofuran. The inhibition percentage was calculated according to Eq. (1).

Statistical methods

The precision and reproducibility of the constructed biosensor were evaluated by measuring the enzyme's activity for 10 consecutive samples. The results were evaluated by descriptive statistics and linear regression methods. All calculations were performed using MATLAB software program in the Windows XP environment. The calculations were obtained by number solving and statistical processing data using the functions polyfit and polyval.

Results and discussion

Sensitivity of the biosensor to acetylcholine

The hybrid membrane ETMS with CAPH and copolymer between AA/AN was selected for the construction of the optical biosensor for pesticides determination because of its highest relative activity for AChE and ChO enzymes, 85.80% and 72.96% respectively according to our previous work [24].

The sensitivity of the optical biosensor to acetylcholine was determined. The acetylcholine was used as test substrate at different concentrations.

The time response of the optical biosensor at different concentrations of acetylcholine is shown in Fig. 1. Depending on the substrate concentration, the response time is around 90 sec in contacting a static state's evidence, and the operating stability is 25 days. Working linear range of the acetylcholine is 2.5 - 30 mM. The calibration curve obtained by the constructed optical biosensor is shown in Fig. 2.

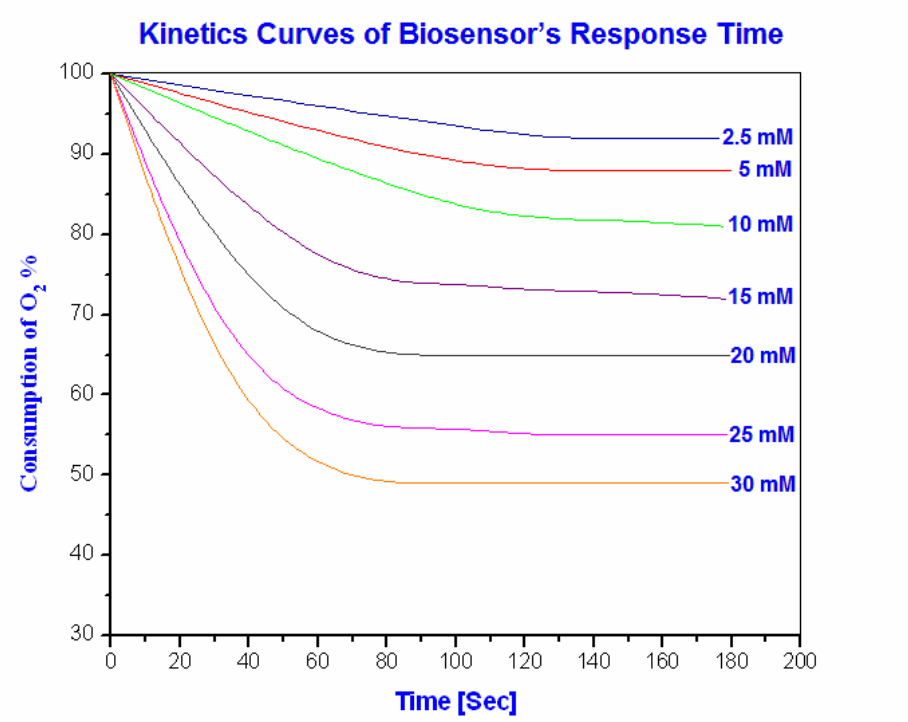

Fig. 1 Response time of the optical biosensor at different acetylcholine concentrations

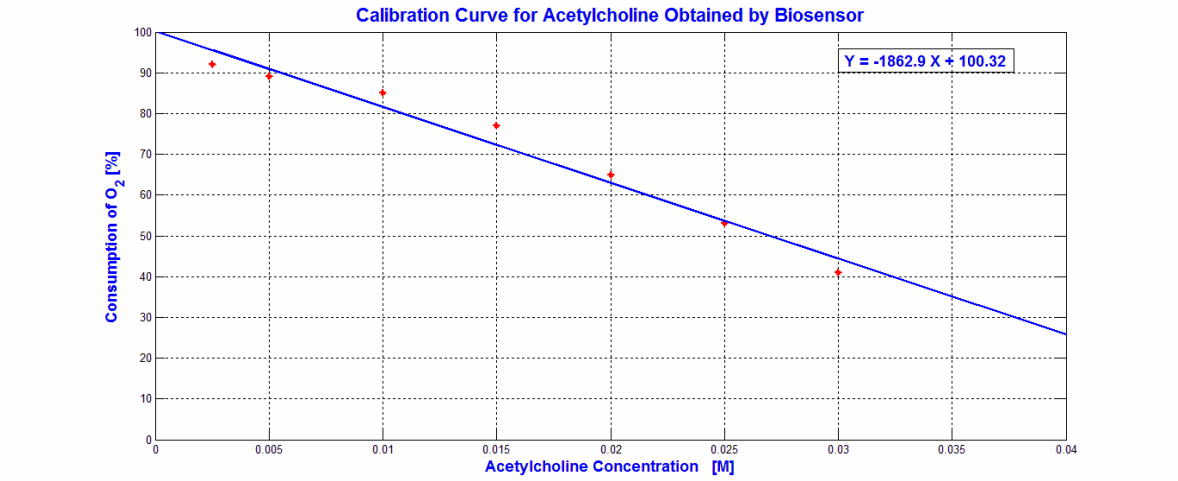

Fig. 2 The calibration curve of acetylcholine

The statistical analyses of the samples are shown in Table 1 and Table 2. The values of the mean, standard deviation, variance and the relative standard deviation % after measuring the absorbance and oxygen consumption for 10 consecutive samples were calculated.

Table 1. Statistical data of the absorbance measurements obtained by the biosensor

ACh concentration	*n*	Mean	Standard deviation	Coefficient of variation	R.S.D. %	Variance
2.5 mM	10	0.0577	0.00207	0.03788	3.78	0.000004
30 mM	10	0.2462	0.01077	0.046106	4.61	0.000116

Table 2. Statistical data of the O_2 consumption measurements obtained by the biosensor

ACh concentration	*n*	Mean	Standard deviation	Coefficient of variation	R.S.D. %	Variance
2.5 mM	10	0.878	0.0394	0.04735	4.74	0.001556
30 mM	10	0.431	0.0262	0.06419	6.42	0.000689

It was observed that the multi-enzyme system allows a good reproducibility of the optical biosensor using spectrophotometer and using oxygen probe. It was observed that the coefficient of variation (R.S.D. = 3.78%, n = 10) for 2.5 mM acetylcholine after measuring the absorbance, and (R.S.D. = 4.74%, n = 10) for 2.5 mM acetylcholine after measuring the oxygen consumption. These results are close to the results obtained by G. Yang et al. [7]. They demonstrated reproducibility (R.S.D. = 3.5%, n = 5). Also, the response time of their biosensor was 30 sec and it was shorter than ours (90 sec). They developed a novel label-free opto-fluidic ring resonator (OFRR) biosensor for detection of the pesticides. Their OFRR was based on a micro-sized glass capillary whose circular wall forms a ring resonator that supports the whispering gallery modes (WGMs). The WGMs has an evanescent field in the capillary core and interacts with the analyte flowing in the capillary. They demonstrated that AChE enzyme was strongly crosslinked immobilized on the OFRR surface. However, our biosensor was based on fiber optic technique with a simpler construction and the AChE enzyme was covalently immobilized on a novel hybrid membrane.

The results obtained by Doong et al. [6] demonstrated that the response time of the developed biosensor to acetylcholine was highly reproducible (R.S.D. = 3.5%, n = 8). These results were also close to our results. They also obtained a linearity of acetylcholine in the range 0.5 - 20 mM. It is more sensitive in small concentrations but our linearity range of acetylcholine is wider (2.5 - 30 mM). They developed a rapid approach for preparing sol-gel based fiber-optic biosensor with encapsulated pH-sensitive fluorophore-acetylcholinesterase conjugates for the determination of acetylcholine. In comparison, our biosensor has a simpler construction with novel hybrid membrane and has wider linearity range.

The results obtained by Yannis et al. [3] show a reproducibility (R.S.D. = 3 - 5%) in a larger range than our results for absorbance measurements (R.S.D. = 3.78%). They demonstrated that the immobilization was obtained in thick three-layer sandwich material immobilized by crosslinking with glutaraldehyde. However, our biosensor was constructed with a thin layer hybrid membrane.

In all these investigations matrices and methods of immobilization are very widely used. It can be concluded that the optical biosensor offers good precision and reproducibility of the measurements; and the obtained new hybrid membrane and method of immobilization are successfully applied for fiber optic biosensor. Also, it can be concluded that fluorescence fiber optic biosensors for oxygen measurements are more versatile than electrochemical biosensors for their flexibility in design and signal transduction. Optical sensors have advantages in terms of response time, costs and size.

Carbofuran detection studies

The inhibition of acetylcholinesterase is normally attributed to the acylation of the serine-OH in the active site of the enzyme by these compounds. The reaction with acetylcholinesterase is analogous to the enzyme-substrate reaction, whereby a Michaelis enzyme-pesticide complex is first formed, followed by the transfer of the pesticide acyl groups to the serine-OH of the enzyme, and the concomitant release of the side product HB. However, unlike the acylated ester formed from the choline ester, the rate of hydrolysis of the carbamylated acetylcholinesterase was very slow [4]. The factors affecting the inhibition were investigated.

The response of the acetylcholinesterase-based optical biosensor to different concentrations of carbamate (carbofuran) was determined. Carbofuran solution was prepared, and standard solutions were prepared by appropriate dilution in (7.5 mM) Tris buffer solution (pH 7.5).

The corresponding calibration curve for carbofuran is illustrated in Fig. 3. The plot appears sigmoidal and exhibits linear response in the concentration range 5×10^{-8} - 5×10^{-7} M for carbofuran in the presence of different concentrations of acetylcholine. The detection limit is taken as the concentration at which the inhibition is 10% was 2.1×10^{-8} M for carbofuran. It was established that this linear range is similar to the range (5×10^{-8} - 5×10^{-7} M) reported by Narayanaswamy [4], and it is close to the range (8×10^{-8} - 1.5×10^{-7} M) reported in [22].

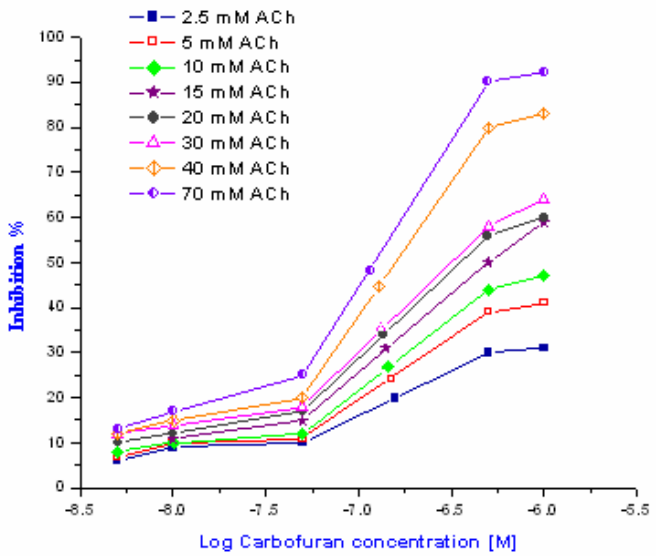

Fig. 3 Calibration curves for different carbofuran concentrations

The inhibition constant (K_i) values of free and immobilized AChE were determined. The results showed that carbofuran acts as uncompetitive inhibitor for free and immobilized

AChE enzyme. The K_i values for 100 nM carbofuran for the immobilized and the free AChE are 1.852×10^{-6} M and 0.8709×10^{-6} M respectively. It can be seen that the free AChE exhibited the lowest K_i value which means that free AChE possesses a higher affinity toward carbofuran than the immobilized AChE.

In [22] are developed amperometric acetylcholinesterase biosensors for quantification of the pesticides carbofuran, carbaryl, methylparaoxon. They demonstrated that enzymes were immobilized on cobalt(II) phthalocyanine-modified electrodes by entrapment in a photocrosslinkable polymer. The K_i value measured for their AChE biosensor was 4×10^{-6} M, this value was higher than our results, which means that our result possesses a higher affinity toward carbofuran. This is mainly due to the covalent immobilization and the minimizing of mass transfer process, compared with entrapment immobilization methods.

Our results (0.8709×10^{-6} M for free AChE and 1.852×10^{-6} M for the immoibilized AChE) are better than the results obtained by Herzsprung et al. [11]. They reported that the K_i values were 1.8×10^{-6} M for the soluble AChE and 0.7×10^{-6} M for immobilized enzyme. They selected organophosphorus and carbamates for determination of their inhibition values (rate constants K_i) on immobilized acetylcholinesterase and butyrylcholinesterase. Our results showed that the free AChE exhibited the lowest K_i value which means that free AChE possesses a higher affinity toward carbofuran.

It can be concluded that our biosensor for carbofuran detection exhibits good sensitivity for carbofuran where the detection limit was 2.1×10^{-8} M, with a wide linearity range in the concentration range 5×10^{-8} - 5×10^{-7} M. Also, it can be concluded that fluorescence fiber optic biosensors for oxygen measurements are more versatile than electrochemical biosensors for their flexibility in design and signal transduction. Optical sensors have advantages in terms of response time, costs and size. In addition, fibers can be miniaturized, which is suitable for in vivo applications.

Conclusion

The fiber-optic biosensor based on simultaneous covalent immobilization of acetylcholinesterase and choline oxidase on a novel hybrid membrane for pesticides detection was constructed. It has been confirmed that the AChE activity depends on the type of the hybrid membranes. By studying the catalytic properties and inhibition of soluble and insoluble AChE, it was found that the ETMS with CAPH and AA/AN membrane constitutes the best system for the optical biosensor construction. This novel hybrid membrane has the highest activity in the absence of inhibitor and the highest inhibition when incubated in the presence of carbofuran. The designed optical biosensor based on a novel hybrid membrane demonstrated high stability (25 days), short response time (90 sec), and wide linear working range of the acetylcholine (2.5 - 30 mM). Inhibition measurements using carbofuran as representative of carbamate pesticides proved successful. The calibration curves for carbofuran exhibited linear response in the concentration range (5×10^{-8} - 5×10^{-7} M). The detection limit measured at $I_{10\%}$ was 2.1×10^{-8} M for carbofuran. The regeneration of carbamate-inhibited enzyme was simple and only entailed treatment with the substrate solution. It can be concluded that our optical biosensor offers good precision and reproducibility of the measurements; and the obtained new hybrid membrane and method of immobilization are successfully applied for fiber optic biosensor.

Acknowledgements

The presented work is supported by project DUNK 01/3 "Scientific Research" Fund, Republic of Bulgaria.

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Prof. Lyubov Yotova, Ph.D.
E-mail: LYotova@tea.bg

Professor Dr. Lyubov Yotova, Department of Biotechnology, E.C.T.S Expert of the Faculty of Chemical and System Engineering of University of Chemical Technology and Metallurgy, Sofia. *Professional Teaching Experience Areas*: Biotransformation Analyses of bioproducts, Biosensors, including guest-professor at the European Biotechnology Master Course at the University of Perugia, Italy. *Scientific and research interests*: Biotransformations with immobilized biocatalysts for industrial and environmental application. Biosensors for medicine, foods and environmental pollutants. Total number of publications 82, 1 textbook. Research projects – 18, at the moment supervisor of 3 National with international participation. Coordinator of NATO project 2000-2003 with the University of Manchester. Coordinator of 3 projects ERASMUS Networks in Biotechnology. Coordinator of 6-th FP project Marie-Curie action. At the moment workpackage leader of "Biosensor application in medicine, food and environment", project No 134310-LLP-2007-1-IT ERASMUS-ENW – EUROBIOTECH (2007-2010).

Nourelhoda Medhat, M.Sc.
E-mail: bio_eng_nour@yahoo.com

Nourelhoda Medhat graduated in 2004 from Biomedical Engineering Department – Helwan University, Cairo, Egypt. She is working as a lecturer assistant in Biomedical Engineering Department – Minia University, Minia, Egypt. She awarded her M.Sc. from Biomedical Engineering and Systems Department – Cairo University, Cairo, Egypt. Currently, she is Ph.D. student in Biotechnology Engineering Department – University of Chemical Technology and Metallurgy, Sofia, Bulgaria. She has interests in Biotechnology and Biosensors applications.