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A fluorescent biosensor based on acetylcholinesterase and 5-oxazolone derivative immobilized in polyvinylchloride (PVC) matrix

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Abstract

A new 2-phenyl-4-[4-(1,4,7,10-tetraoxa-13-azacyclopentadecyl)benzylidene]-5-oxazolone (CPO) derivative was utilized to develop an optical acetylcholinesterase (AChE) biosensor in which the azlactone derivative was embedded in plasticized polyvinylchloride (PVC) matrix. The sensor system was calibrated for the detection of acetylcholine (ACh) and donepezil which is a competitive cholinesterase (ChE) inhibitor. Two different biosensing systems were developed by using AChE enzyme in solution and immobilized together with the fluorescent derivative (CPO) doped in transparent PVC membrane. The enzymatic hydrolysis of ACh was monitored by following changes in the pH induced fluorescence intensity. When AChE enzyme was immobilized in PVC matrix together with CPO, the sensitivity of the measuring system has increased approximately three times for ACh, in comparison to the sensing system where AChE enzyme was in solution phase.

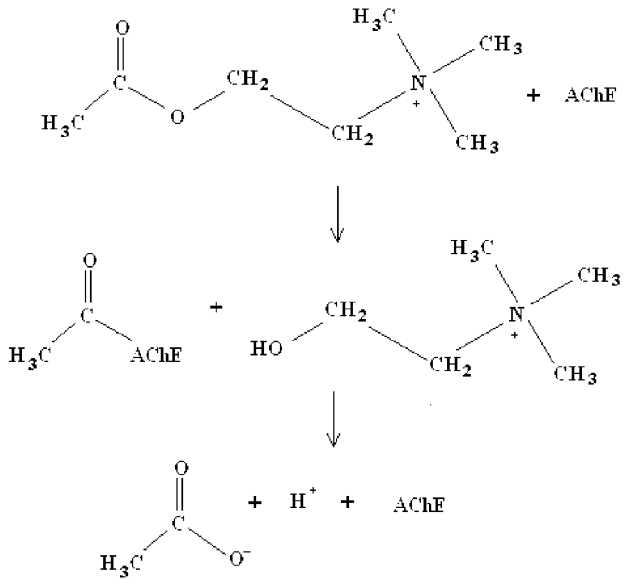
The photophysical parameters of CPO were also examined in solvents of tetrahydrofuran (THF), acetonitrile (ACN) and dichloromethane (DCM) and in solid matrix of PVC. The azlactone derivative exhibits excellent photostability in PVC matrix.

Keywords: Acetylcholine; Donepezil; 5-Oxazolone; Fluorescence emission; Polymer film

1. Introduction

Extensive studies on 5-oxazolone dyes have shown favourable photophysical and photochemical properties in crystalline state, which resulted in their use in semiconductor devices such as electrophotographic photoreceptors, and in non-linear optical materials [1,2]. The luminescence properties of azlactone dyes have been investigated and very low fluorescence efficiencies have been reported for solutions in different solvents [1–5]. It was previously reported that immobilization of 5-oxazolone molecules resulted in higher fluorescence emission yields as well as improved stability toward chemical and photochemical environment [1]. Hence, dye molecules in PVC matrices provide a good alternative for sensor applications.

Cholinesterase biosensors are used for different purposes such as pesticides, drugs and neurotransmitter detection [6–33]. ACh is a biomolecule which plays an important role in nerve impulse transmission in the peripheral and central nervous systems thus its detection is of major importance in biotechnology as well as in clinical applications. AChE is capable of hydrolysing the ACh biocatalytically to acetic acid and choline. The hydrolysis of ACh occurs in three different steps and may be expressed by the following reactions [33]:



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First, the enzyme and the substrate combine to form the enzyme–substrate complex. Then the acetyl group transfers to the serine group in the active site of the esterase molecule to form the acetylated enzyme and choline chloride (ChCl). The third step involves the rapid hydrolysis of the acetylated enzyme to produce the free enzyme and acetic acid which is the second hydrolytic product. Acetic acid release results in a decrease of the pH in the local environment of the enzyme which could be also called as bioactive region. For monitoring the reaction optically, the formation of the proton resulting from enzymatic activity can be followed by transduction of pH decrease to an optically measurable signal by immobilized pH indicator.

Different ChE biosensors were previously developed for the pesticide, drug or neurotransmitter detection in different matrices. Wong et al. reported the construction of an optical biosensor based on a lipophilic chromoionophore doped in sol–gel films for the determination of dichlorvos inhibition, an organophosphate insecticide [8]. Moreover, Hai et al. developed a bioelectronic hybrid system for the detection of ACh by immobilizing the AChE enzyme to the gatesurface of anion sensitive field-effect transistor (ISFET) [9]. An amperometric biosensor for the detection of carbamate pesticides based on inhibition of ACh immobilized by entrapment in an optimized sol–gel matrix on screen-printed electrodes was developed by Bucur et al. [10]. Furthermore, Lenigk et al. presented an electrochemical method for the investigation and comparison of anti-Alzheimer medication that is based on the inhibition of the AChE [28].

Liposome-based biosensors were prepared by encapsulating the AChE in L-phosphatidylcholine liposomes resulting in spherical optical biosensors [14]. The enzyme activity within the liposome was monitored using pyranine, a fluorescent pH indicator. Schuvailo et al. developed a carbon fibre-based ACh micro-biosensor for *in vivo* neurotransmitter measurements [15]. A potentiometric AChE biosensor based on a pH-sensitive PVC matrix membrane with plasma-polymerized ethylenediamine film was prepared by Liu et al. [17]. Choi et al. developed a fiber-optic biosensor consisting of an AChE-immobilized Langmuir–Blodgett film to detect organophosphate compounds in contaminated water [26]. The sensing scheme was based on the decrease of yellow product, *o*-nitrophenol, from a colourless substrate, *o*-onitrophenylacetate, due to the inhibition by organophosphate compounds. In addition to the mentioned biosensing systems, rapid approaches for preparing sol–gel based fiber-optic biosensors were developed for both neurotransmitter and insecticide analysis by Doong and Tsai [27] and Xavier et al. [30].

Donepezil hydrochloride ((±)-2-[(1-benzyl-piperidine-4-yl)ethyl]-5,6-dimetoxyindan-1-one hydrochloride), is a potent, selective and reversible AChE inhibitor and has been prescribed worldwide for the treatment of Alzheimer's disease which is a neurodegenerative disorder characterized by progressive loss of memory followed by complete dementia [34,35]. More recently, donepezil treatment for Down syndrome showed potential improvement of the symptom in a non-randomized-controlled trial. In addition some reviews on the pharmacokinetics, pharmacodynamics and clinical profiles of donepezil have been published [36–38]. In these views, the determination of donepezil in biological fluids has become significant, and thus the development of a simple and sensitive method for determining donepezil is required. HPLC methods have been generally reported for determination of donepezil in tablets and plasma [34,35,39–41]. Gotti et al. previously, described the analysis of donepezil by capillary electrophoretic method based on a rapid migration of the analyte obtained under acidic conditions [42].

Here we present a new optical thin-film biosensor based on the fluorescent pH sensitive azlactone dye entrapped in PVC matrix. The presented work is composed of two parts; first, the azlactone dye; 2-phenyl-4-[4-(1,4,7,10-tetraoxa-13-azacyclopentadecyl)benzylidene]-5-oxazolone (CPO) was synthesized and characterized and then, applied as a part of biosensing system for both neurotransmitter and inhibitor analysis.

Data, obtained from the system based on the fluorescent CPO entrapped in PVC matrix together with enzyme AChE, were compared with the other system in which the enzyme was used in a soluble form in buffer media instead of immobilized form together with the dye in the solid matrix. It is clearly demonstrated that, the protective PVC matrix contributes to the stabilization of the sensor slides inhibiting bending of the enzyme and providing a rigid microenvironment for the fluorescent molecule. The sensor slides were calibrated for donepezil which belongs to a new class of AChE inhibitors as well as the ACh neurotransmitter. Sensor characteristics such as response time, reversibility and reproducibility were tested and evaluated. In addition, the photophysical characteristics of CPO in three different solvents of THF, ACN and DCM and in solid matrix of PVC were determined.

2. Experimental

2.1. Materials

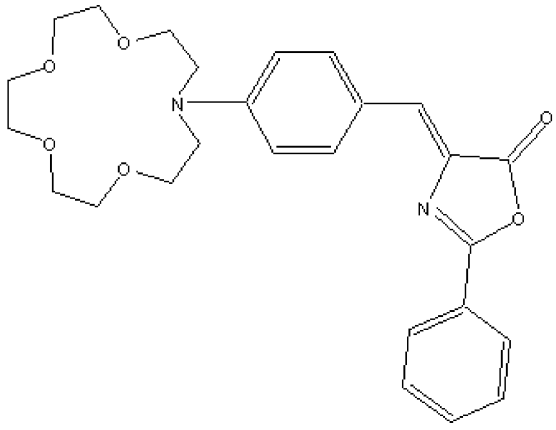
The synthesis and purification of 2-phenyl-4-[4-(1,4,7,10-tetraoxa-13-azacyclopentadecyl)benzylidene]-5-oxazolone (CPO) were performed by using general preparation methods of azlactones as described in the literature [5].

Acetylcholinesterase (AChE; EC 3.1.1.7, from Electric EL, 500 U/mg) and acetylcholine chloride were obtained from Sigma. The membrane components, PVC (high molecular weight) and the plasticizer bis-(2-ethyl-hexyl)phthalate (DOP) were supplied by Fluka, Lipophilic anionic derivative reagent potassium tetrakis-(4-chlorophenyl)borate (PTCPB), tetrahydrofuran (THF), acetonitrile (ACN) and dichloromethane (DCM) were obtained from Aldrich. All other chemicals were obtained from Fluka and Merck. The polyester support (Mylar type) was provided from DuPont, Switzerland. Bidistilled ultra pure water was used throughout the studies.

^{1}H NMR of CPO ($CDCl_3$, 400 MHz, δ (ppm)): 3.54–3.84 (m, 20H, –N–CH_2OCH_2–), 7.17 (s, 1H, –CH=C–(C=O)–), 7.5–8.05 (m, 5H, –C_6H_5), 6.82–6.92, 8.19–8.24 (d, d, 2H, 2H, –N–CH=CH–, –N–CH=CH–).

Other selected analytical data for CPO are shown below:

Compound	MW (g/mol)	Color	mp (°C)	Isolated yield (%)	ν (cm^{-1})				
					(–O–C=O)	(O–C=O)	(CH_2–CH_2–O–)	(–C=N–)	(–C–N)
CPO	466.53	Orange	159	21	1764	1160	1123	1639	1604



CPO

2.2. Polymer film preparation

The polymer films were prepared from a mixture of 120 mg of PVC, 240 mg of plasticizer, equimolar PTCPB to dye CPO (2×10^{-6} mol CPO/kg PVC) and 1.5 mL of THF (dry). Finally, AChE (100 unit of AChE in 10 μL of distilled water) was added, stirred properly and the resulting cocktail was spread onto a 125 μm polyester support (Mylar type). PCV films were kept in refrigerator at 4 °C. Each sensor PVC film was cut with width of 12 mm.

2.3. Apparatus and measuring procedures

The absorption spectra of PVC slides were measured by using Shimadzu UV-1601 spectrophotometer. All fluorescence measurements were recorded by using Varian-Cary Eclipse spectrofluorimeter. The pH values of buffer solutions were adjusted with WTW pH-meter calibrated with Merck pH standards of pH 7.00 (titrisol) and pH 4.01. IR spectra were recorded on a Perkin Elmer Spectrum BX FTIR spectrometer as KBr pellets, while ^{1}H NMR spectra were recorded on a Varian Mercury AS 400 NMR spectrometer at 400 MHz.

Absorption and fluorescence emission spectra of PVC membranes were acquired in quartz cells which were filled with sample solution. The polymer films were placed in diagonal position in the quartz cell. The advantage of this kind of placement was to improve the reproducibility of the measurements.

In the present study, ACh was used as substrate of AChE which catalyzes the hydrolysis of the substrate to choline and acetic acid. The release of acid results in a decrease of the pH of the bulk electrolyte solution. This decrease was transduced to an optically measurable signal by the immobilized dye. The analytical signal corresponds to the rate of decrease in fluorescence intensity depending on the acetic acid concentration released as a result of enzymatic hydrolysis of the substrate.

In case of AChE inhibitor (donepezil) detection, measurements were carried out in the presence of donepezil and a decrease in florescence intensity due to the competitive inhibition was observed. These signals were plotted versus donepezil concentrations. During these experiments constant substrate concentration at saturation level which was chosen from the calibration curve of ACh was used to keep all the enzymes in the active form. Since competitive inhibition occurred, no regeneration step was required before the next measurements.

In the reaction, 2.5 mM phosphate buffer (pH 7.0) was chosen as a working buffer and AChE was used in a soluble form in working buffer solution (only CPO immobilized in PVC matrix) (a), and also in immobilized form in PVC matrix together with CPO (b).

Enzyme-substrate response curves were obtained by adding a known amount of ACh substrate in 3 mL of reaction mixture (2.5 mM, pH 7.0) containing 4 unit enzyme activity in quartz cell (a), and by adding a known amount of ACh in 3 mL of working buffer solution (2.5 mM, pH 7.0), (4 unit AChE activity immobilized in PVC matrix) in quartz cell (b). Both results obtained for the soluble and immobilized enzyme were evaluated and compared with each other. All measurements were performed at ambient conditions and the decrease in fluorescence intensity depending on the substrate concentration was recorded with time for 4 min if otherwise not stated.

3. Results and discussion

3.1. UV–vis absorption and fluorescence emission spectroscopy studies

Absorption and fluorescence emission based spectral data are given in Table 1. The absorption maxima of CPO were at the wavelengths of 474, 469 and 473 nm in the solvents of DCM, ACN and THF, respectively. Wavelength of the absorption, in PVC film is seen to be shifted about 22–27 nm to shorter wave-

Table 1
UV–vis absorption and emission spectroscopic data, λ_{abs}^{max} (nm), λ_{emis}^{max} (nm), Stokes' shift, $\Delta\lambda$ (nm), molar extinction coefficients, ε_{max} (L mol^{-1} cm^{-1}) and singlet energy, E_s (kcal/mol) of CPO in solvents of DCM, ACN and THF and PVC matrix

Solvent	λ_{abs}^{max}	λ_{emis}^{max}	$\Delta\lambda$	ε_{max}	E_s
DCM	474	522	48	76,000	60.2
CAN	469	536	67	88,000	60.9
THF	473	523	50	127,000	60.3
PVC film	447	515	68	125,000	63.8

length in comparison to the solution phase. This blue shift of absorption of CPO may be related to the decrease of the polarity of microenvironment of CPO.

In all of the employed solvents and PVC matrix, the excitation wavelength was chosen as 445 nm and emission spectra were recorded. The CPO dye exhibited moderate results concerning with Stokes' shift values which range from 48 to 68 nm in all of the employed media. The highest Stokes' shift value was observed in PVC matrix, which confers the advantage of better spectral resolution in emission based studies and a desired property in commercially important dyes.

Singlet energy of the CPO in solutions is observed to be lowered about 2.9–3.6 kcal mol^{-1}, with respect to E_s of CPO in polymer film matrix $E_s = 63.8$ kcal mol^{-1}.

The photostability of CPO in ACN, DCM and THF and in solid matrix of PVC were determined with a steady-state spectrofluorimeter in time based mode. CPO was excited at 445 nm and the data were acquired at its maximum emission wavelength in ACN, DCM and THF and in solid matrix of PVC. The data collected after 1 h monitoring shown in Fig. 1, reveals the excellent photostability of CPO in PVC matrix, but lower photostability in ACN, DCM and THF.

3.2. Optimization of biosensor

3.2.1. pH effects on signal intensity

The pH dependence of buffer solution on sensor system in the range of 6.0–8.2 was investigated both in plain buffers of 2.5 mM phosphate (pH 6.0, 6.5, 7.0, 7.5, 8.0 and 8.2) and in the presence of 0.66 mM ACh; substrate containing buffers. A decrease in relative signal change was observed above pH 8.0. This can be attributed to the loss of enzymatic activity which results in less proton formation. Thus, pH 7.0 was chosen as an appropriate measurement medium. In order to avoid the other interfering pH dependent signal fluctuations, we recorded all of the spectra in phosphate buffered solutions.

3.2.2. Response time

The response time of the sensor membrane was investigated in 2.5 mM pH 7.0 phosphate buffer after addition of 0.66 mM ACh. Since maximum change in fluorescence intensity due to the enzymatic hydrolysis of the substrate was obtained after 4 min and remained constant for further increments in the time, the optimum response time of the sensor system was chosen as 4 min.

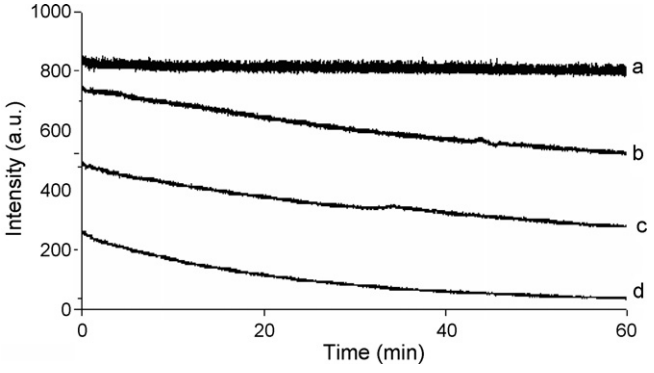


Fig. 1. Photostability test results of CPO in (a) PVC (2×10^{-6} mol CPO/kg PVC), (b) THF (10^{-6} M CPO, $\lambda_{max} = 473$ nm), (c) DCM (10^{-6} M CPO, $\lambda_{max} = 474$ nm) and (d) ACN (10^{-6} M CPO, $\lambda_{max} = 469$ nm) after 1 h of monitoring.

3.3. Characterization studies

Sensor slides respond to proton resulting from enzymatic activity. The release of the acid anion results in a decrease of the pH of the bulk electrolyte solution. This decrease was transduced to an optically measurable signal by the immobilized dye. The analytical signal corresponds to the rate of decrease in fluorescence intensity. The sensor response was monitored for two different systems. In the first measuring system CPO was entrapped in PVC matrix and enzyme was free in buffer media. In the second measuring system enzyme AChE was entrapped in PVC matrix together with CPO.

3.3.1. The system with the soluble AChE

Fluorescence intensity based response of CPO in PVC in the presence of soluble enzyme for ACh in the concentration range of 0.33–3.23 mM ACh is given in Fig. 2(a).

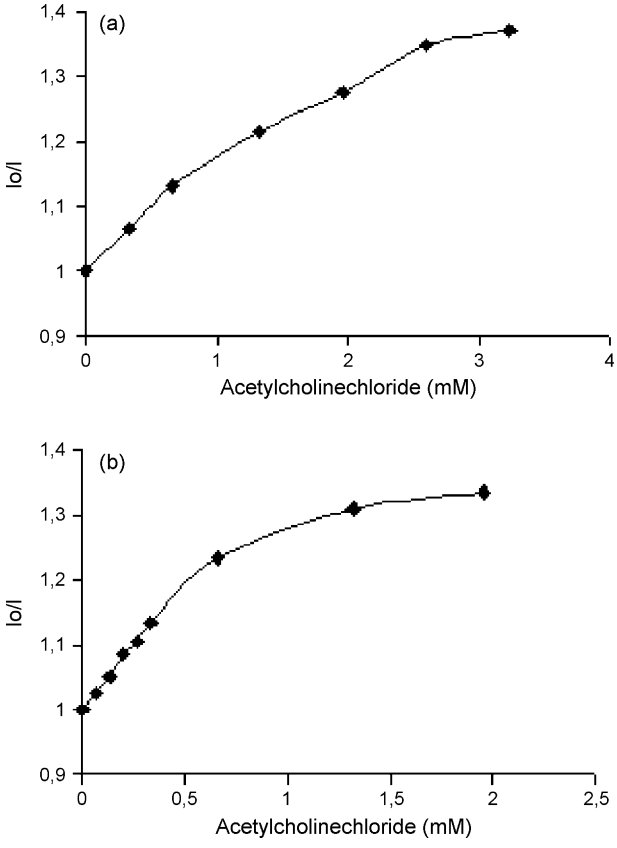


Fig. 2. Intensity based response curve of CPO in (a) PVC (2×10^{-6} mol CPO/kg PVC) in 2.5 mM pH 7.0 phosphate buffer containing 4 unit AChE activity/3 mL in the concentration range of 0.33–3.23 mM acetylcholine chloride and (b) PVC (2×10^{-6} mol CPO/kg PVC, 4 unit AChE activity) in 2.5 mM pH 7.0 phosphate buffer in the concentrate range of 0.07–2.0 mM ACh.

3.3.2. The system with immobilized AChE in PVC matrix

The response of the biosensor when AChE and CPO are immobilized together in PVC matrix for ACh in the concentration range of 0.07–2.0 mM is shown in Fig. 2(b). According to our findings, more sensitive system is obtained in comparison to the other one in which AChE is in solution phase.

A linear relationship between sensor responses and ACh concentration was obtained in concentration range between 0.33 and 2.6 mM, for the system in which the enzyme was dissolved in solutions, with a linear calibration plot defined by the equation $y = 0.1297x + 1.02$ and $R^2 = 0.982$. On the other hand, when AChE enzyme was immobilized in PVC matrix together with CPO, the sensor response of the sensing system has improved to concentration range between 0.07 and 0.66 mM with linear calibration plot defined by the equation of $y = 0.355x + 1.01$ and $R^2 = 0.993$.

The limit of detection (LOD) for ACh was defined as the concentration at which the signal is equal to the average blank signal ± 3 standard deviation ($n = 5$). LOD value for ACh was found to be 0.043 mM when AChE enzyme was in immobilized form.

As well as detection linearity, LOD value of the proposed system for ACh was found to be sub mM level and exhibited better sensitivities in comparison to the previous works in which a sol–gel based fiber-optic biosensor with encapsulated pH-sensitive fluorophore-AChE was developed [27] and an optical sensor containing a chromoionophore doped sol–gel film interfaced with another sol–gel film immobilized with AChE [8]. In addition, other optical sensor based on ChE/liposome was depicted previously and this was found to be less sensitive towards to AChE substrate; ATChCl which was at mM level [14]. The development of an optical fiber biosensor based on AChE inhibition and pH transduction was also described by Xavier et al. and applied for the detection of carbamate insecticides and a linear calibration plot for ACh was obtained in the range of 0.1 and 1.5 mM and this sensor allows lower detection limits for carbamate pesticides; propoxur (0.4 ng) and carbaryl (25 ng) [30].

3.3.3. Reproducibility and reversibility

The reproducibility and reversibility of the optical responses were assessed by repeatedly introducing a sample of 0.13 mM ACh and a phosphate buffer at pH 7.0 under batch conditions by using the system including immobilized dye and enzyme together in the PVC matrix (Fig. 3). Between second and the fifth cycles, the level of reproducibility achieved corresponded to a R.S.D. of 0.3. The sensor was fully reversible and a positive drift of 0.15% of the upper signal has been observed after the first cycle. The following second and third cycles did not result in any further large drifts. Fluorescence intensity of the dye is found to decrease about 0.29% after fourth cycle. The results indicate that the reversibility of the sensor slides is satisfactory.

3.4. Sensor response for AChE inhibitor; donepezil

As is already mentioned in the measuring principle, donepezil treatment caused inhibition of the enzyme activity, hence lower

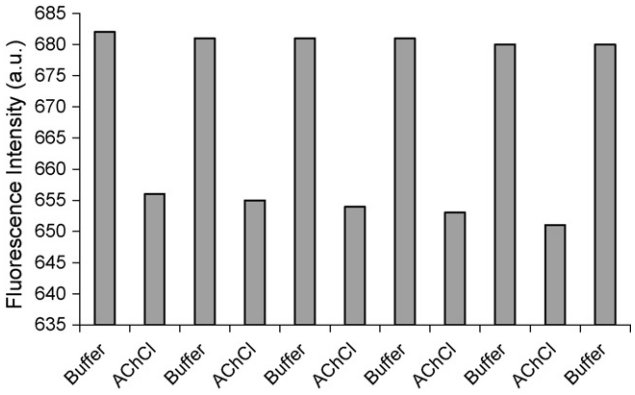


Fig. 3. Reproducibility of CPO to 0.13 mM ACh in 2.5 mM pH 7.0 phosphate buffer in PVC (2×10^{-6} mol CPO/kg PVC, 4 unit AChE activity) for five measurements.

decrease in fluorescence intensity was observed. Inhibition levels were found to be well correlated with donepezil concentration. Fig. 4 shows the emission spectra of CPO in PVC (2×10^{-6} mol CPO/kg PVC, 4 unit AChE activity) in 2.5 mM pH 7.0 phosphate buffer containing 2.0 mM ACh in the concentration range of 2.35×10^{-5} to 43.63×10^{-5} M donepezil. Furthermore, intensity based response curve of CPO in PVC (2×10^{-6} mol CPO/kg PVC, 4 unit AChE activity) was also given in Fig. 4. Linearity was observed in the range of 2.38×10^{-5} and 43.63×10^{-5} M and linear graph was defined by equation of $y = 0.031x + 0.9445$ with the correlation coefficient of $R^2 = 0.995$.

LOD value for Donepezil was defined as the concentration at which the signal is equal to the average blank signal ± 3 standard deviation ($n = 5$) and found to be 1.01×10^{-5} M.

The reproducibility and reversibility behaviour of the sensor composition were tested by repeatedly exposing the sensor slides to donepezil concentration of 9.41×10^{-5} M in 2.5 mM at pH 7.0 phosphate buffer containing 2.0 mM ACh. At each time the reagent phase was regenerated by a phosphate buffer at pH 7.0.

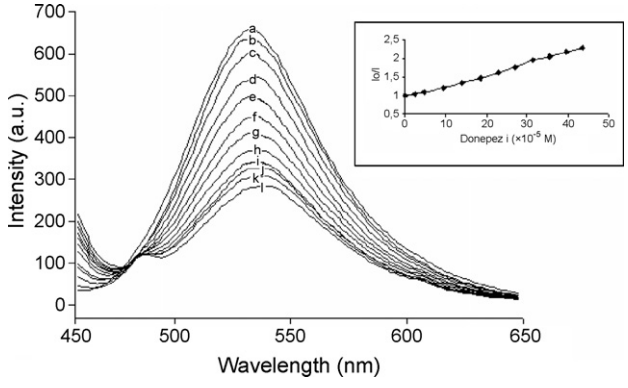


Fig. 4. Emission spectra of CPO in PVC (2×10^{-6} mol CPO/kg PVC, 4 unit AChE activity) in 2.5 mM pH 7.0 phosphate buffer containing 1.96 mM acetylcholine chloride after exposure to different donepezil concentrations (a) 0, (b) 2.38, (c) 4.75, (d)9.41, (e) 13.98, (f) 18.46, (g) 22.85, (h) 27.16, (i) 31.40, (j) 35.55, (k) 39.63 and (l) 43.63×10^{-5} M and intensity based response curve of CPO in PVC (2×10^{-6} mol CPO/kg PVC, 4 unit AChE activity) in 2.5 mM pH 7.0 phosphate buffer containing 2.00 mM acetylcholine in the concentrate range of 2.38×10^{-5} to 43.63×10^{-5} M donepezil.

The relative standard deviation was calculated for upper signal and found to be 1.5% for five measurements. The sensor system was found to be fully reversible.

An electrochemical sensor incorporating AChE and choline oxidase bienzymatic system for the screening of anti-Alzheimer medications based on the inhibition of the AChE was investigated by Leingk et al. [28]. The sensor system has been applied to determine the IC_{50} values of Alzheimer remedy. Here we detected donepezil.

3.5. Sensor stability

Sensor stability was tested in plain buffers of 2.5 mM phosphate (pH 7.0) and in the presence of 0.33 mM acetylcholine chloride containing buffers for 25 consecutive days of measurements. Sensor films were kept in a desiccator in refrigerator at 4 °C. The decreases in the fluorescence intensities in plain buffers of 2.5 mM phosphate (pH 7.0) were measured as 0.1, 0.2, 0.6, 0.9, 1.5 and 2.0% after the 2nd, 3rd, 6th, 12th, 18th and 25th days, respectively. In the presence of 0.33 mM ACh containing buffers, the decreases in the fluorescence intensities were as 0.3, 0.7, 1.2, 1.5, 2.0 and 2.7% after the 2nd, 3rd, 6th, 12th, 18th and 25th days, respectively. The results obtained indicate that the stability of the sensor system is satisfactory.

4. Conclusions

The construction and characterization of an optical biosensor based on AChE and CPO dye immobilized in PVC matrix have been described and its application to acetylcholine and donepezil detection has been investigated. Higher sensitivity (approximately three times) for ACh was obtained by using immobilized AChE with CPO in PVC, when compared with the other system in which soluble enzyme was used. The enzyme probe can be used for ACh sensing in concentration range of 0.07–2.0 mM. Sensor slides exhibited response toward donepezil in the concentration range of 2.38×10^{-5} to 43.63×10^{-5} M. The sensor system was fully reversible and reproducible, further meriting that fluorescent CPO derivative may be an alternative indicator for enzymatic neurotransmitter and donepezil sensing.

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