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Photophysical characterization of fluorescent oxazol-5-one derivatives in PVC and their application as biosensors in the detection of ACh and AChE inhibitor: Donepezil

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Abstract

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 acetylcholinesterase, have been developed. The biosensors were calibrated for acetylcholine as neurotransmitter and the potent, selective and reversible cholinesterase inhibitor, *donepezil* ((±)-2-[(1-benzyl-piperidine-4-yl)ethyl]-5,6-dimetoxyindan-1-one hydrochloride). The photophysical parameters of all derivatives were also examined in PVC in which medium, they exhibited excellent photostability.

Keywords: Acetylcholinesterase; Neurotransmitter; Donepezil; 5-Oxazolone; Fluorescence emission; Biosensor

1. Introduction

Cholinesterase biosensors are used for different purposes such as the detection of pesticides, drugs and neurotransmitters [1–27]. As acetylcholine (ACh) is a biomolecule which plays an important role in nerve impulse transmission in the human peripheral and central nervous systems, its detection is of major importance in biotechnology and clinical applications. Acetylcholinesterase (AChE) is capable of hydrolysing ACh biocatalytically to acetic acid and choline via three different steps [27]:

$$\underset{\text{AChCl}}{CH_3COOCH_2CH_2N^+(CH_3)_3Cl^-} \overset{\text{AChE}}{\rightleftharpoons} \underset{\text{ACh-AChE complex}}{[CH_3COOCH_2CH_2N^+(CH_3)_3Cl^-\text{AChE}]} \rightarrow \underset{\text{Acetylated AChE}}{\text{AChE-OOCHCH}_3} + \underset{\text{ChCl}}{HOCH_2CH_2N^+(CH_3)_3Cl^-}$$

$$\underset{\text{Acetylated AChE}}{\text{AChE-OOCHCH}_3} + H_2O \rightarrow \underset{\text{Acetic acid}}{CH_3COOH} + \text{AChE}$$

The enzyme and the substrate combine to form an enzyme–substrate complex and 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); finally, the acetylated enzyme is rapidly hydrolysed to produce the free enzyme and acetic acid which is the second hydrolytic

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product. Acetic acid release results in a decrease in the pH in the local environment (bioactive region) of the enzyme. To monitor the reaction optically, the formation of the proton resulting from enzymatic activity can be followed using an optically measurable signal from an immobilized pH indicator.

Various cholinesterase (ChE) biosensors have been previously developed using different matrices. Wong et al. reported the construction of an optical biosensor based on sol–gel films doped with a lipophilic chromoionophore for the determination of dichlorvos inhibition, an organophosphate insecticide [2]. Moreover, Hai et al. developed a bioelectronic hybrid system for the detection of ACh by immobilizing the AChE enzyme to the gate surface of an anion sensitive field-effect transistor (ISFET) [3]. An amperometric biosensor for the detection of carbamate pesticides based on the inhibition of ACh immobilized by entrapment in an optimized sol–gel matrix on screen-printed electrodes was developed by Bucur et al. [4]. Furthermore, Lenigk et al. presented an electrochemical method for the investigation and comparison of anti-Alzheimer medication based on inhibition of AChE [22].

Liposome-based biosensors have been prepared by encapsulating AChE in L-phosphatidylcholine liposomes resulting in spherical optical biosensors; the enzyme activity within the liposome was monitored using pyranine, a fluorescent pH indicator [8]. Schuvailo et al. developed a carbon fibre-based ACh micro-biosensor for *in vivo* neurotransmitter measurements [9]. A potentiometric AChE biosensor based on a pH-sensitive PVC matrix membrane using a plasma-polymerized ethylenediamine film was prepared by Liu et al. [11] while Choi et al. developed a fiber-optic biosensor consisting of an AChE-immobilized Langmuir–Blodgett film to detect organophosphate compounds in contaminated water [20]. The sensing scheme was based on the decrease of the yellow product, *o*-nitrophenol, from a colourless substrate, *o*-nitrophenylacetate, due to inhibition by organophosphate compounds. In addition to the above mentioned biosensing systems, rapid approaches for preparing sol–gel based fiber-optic biosensors have been developed for both neurotransmitter and insecticide analysis by Doong and Tsai [21] and Xavier et al. [24].

Moreover, donepezil hydrochloride ((±)-2-[(1-benzyl-piperidine-4-yl)ethyl]-5,6-dimetoxyindan-1-one hydrochloride) is a potent, selective and reversible AChE inhibitor that has been prescribed worldwide for the treatment of Alzheimer's disease [28,29]. More recently, donepezil showed potential improvement of Downe's syndrome in a non-randomized controlled trial. In addition, reviews on the pharmacokinetics, pharmacodynamics and clinical profiles of donepezil have been published from which it is apparent that the determination of donepezil in biological fluids has become significant, for which 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 [28–32]. Gotti et al. previously described the analysis of donepezil by a capillary electrophoretic method based on the rapid migration of the analyte obtained under acidic conditions [33].

Previous work by the current authors described the synthesis of 2,4-diaryl-5-oxazolone derivatives [34]. This paper concerns the synthesis of three, 5-oxazolone derivatives which contain a phenylazacrown-5 moiety at the 4-position and electron donating and withdrawing groups at the 2-position of the oxazolone. The photophysical characterizations of the fluorescent pH-sensitive CPO-I, CPO-II and CPO-III dyes (Scheme 1) entrapped in PVC matrix were carried out using absorption and fluorescence emission spectroscopies and their application as a biosensing system was investigated. Finally, the newly developed optical biosensors were utilized for both neurotransmitter and inhibitor analyses. The sensor slides were calibrated for donepezil which belongs to a new class of AChE inhibitor as well as the ACh neurotransmitter. Sensor characteristics such as response time, reversibility and repeatability were evaluated.

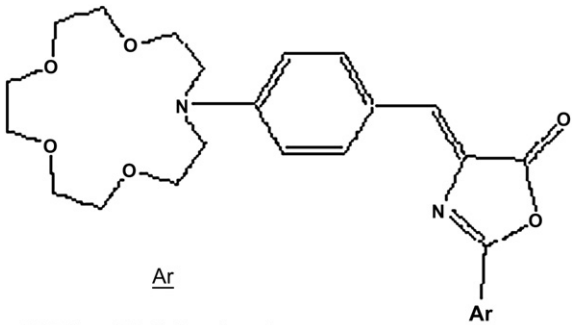
2. Experimental

2.1. Materials

The CPO-I, CPO-II and CPO-III derivatives were synthesized and purified using the general procedure recounted in Ref. [34]. Acetylcholinesterase (AChE; EC 3.1.1.7, from electric eel, 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. The lipophilic anionic reagent, potassium tetrakis-(4-chlorophenyl)borate (PTCPB) and tetrahydrofuran (THF) were obtained from Aldrich. All other chemicals were obtained from Fluka and Merck. The Mylar polyester support was provided by DuPont, Switzerland. Bidistilled ultra pure water was used throughout the studies.

2.2. Polymer film preparation

The polymer films were prepared from a mixture of 120 mg of PVC, 240 mg of plasticizer, equimolar PTCPB:dye CPO (2×10^{-6} mol CPO/kg PVC) and 1.5 mL of THF (dry).



CPO-I 3,5-dinitrophenyl

CPO-II 4-nitrophenyl

CPO-III 4-tolyl

Scheme 1.

Finally, AChE (100 unit of AChE in 10 μL of distilled water) was added with stirring and the resulting mixture was spread onto a 125 μm Mylar polyester support. PCV films were kept in a refrigerator at 4 °C. Each sensor PVC film was cut into slides of 12 mm width.

2.3. Apparatus and measuring procedures

The absorption spectra of the PVC slides were measured using a Shimadzu UV-1601 spectrophotometer; fluorescence measurements were recorded using a Varian-Cary Eclipse spectrofluorimeter. The pH of buffer solutions was adjusted using a WTW pH-meter calibrated with Merck pH standards of pH 7.00 (titrisol) and pH 4.01. The absorption and fluorescence emission spectra of PVC membranes were obtained using quartz cells which were filled with sample solution. The polymer films were placed in a diagonal position in the quartz cell. The use of this placement method improved the reproducibility of measurements.

AChE was used to catalyze the hydrolysis of ACh (the substrate) to choline and acetic acid. The accompanying reduction in pH of the bulk electrolyte solution was converted to an optically measurable signal by the immobilized dye. The analytical signal corresponds to the rate of decrease in the fluorescence intensity depending on the substrate concentration.

In the case of the AChE inhibitor (donepezil) detection, measurements were carried out in the presence of donepezil and, due to the competitive inhibition of the enzyme, as expected the fluorescence intensity was lowered. These signals were plotted as a function of donepezil concentration. During these experiments, a constant substrate concentration (at saturation level), chosen from the calibration curve of Ach, was used to maintain the enzymes in their active form. Since competitive inhibition occurred, no regeneration step was required before subsequent measurements.

Enzyme–substrate response curves were obtained by using a given amount of ACh substrate in 3 mL of buffer solution. All measurements were performed under ambient conditions and the decrease in fluorescence intensity was recorded with a response time of 4 min.

3. Results and discussion

3.1. UV–vis absorption and fluorescence emission spectroscopy studies

Absorption and fluorescence emission based photophysical parameters of CPO-I, CPO-II and CPO-III in PVC matrix were extracted from the spectral data and are given in Table 1. The absorption maxima of CPO-I, CPO-II and CPO-III occurred at 488, 509 and 458 nm in PVC film, respectively (Table 1, Fig. 1).

In the PVC matrix, the excitation wavelengths for CPO-I, CPO-II and CPO-III were chosen as 485, 495 and 450 nm, respectively, and emission spectra were recorded (Fig. 2). The CPO dyes exhibited moderate Stokes' shift values, ranging from 81 to 116 nm which confers the advantage of better spectral resolution in emission based studies.

Table 1
UV–vis absorption and emission spectroscopic data, λ_{abs}^{max} (nm), λ_{emis}^{max} (nm), Stokes' shift, $\Delta\lambda$ (nm) and molar extinction coefficients, ε_{max} (L mol^{-1} cm^{-1}) of CPO-I, CPO-II and CPO-III in PVC matrix

CPO	λ_{abs}^{max}	λ_{emis}^{max}	$\Delta\lambda$	ε_{max}
CPO-I	488	590	102	67,000
CPO-II	509	625	116	142,000
CPO-III	458	539	81	175,500

The photostability of the CPO derivatives in PVC was determined with a steady-state spectrofluorimeter in time-based mode. CPO-I, CPO-II and CPO-III were excited at 485, 495 and 450 nm, respectively, and the data were acquired at their maximum emission wavelengths. According to the data collected after 1 h, there was no change in the fluorescence intensities of the sensor slides.

3.2. Optimization of biosensor

3.2.1. pH effects on signal intensity

The pH dependence of the sensor systems in the range 6.5–8.2 was investigated both in plain buffers of 2.5 mM phosphate (pH 6.5, 7.0, 7.5, 8.0, 8.2) and in the presence of 8.33×10^{-5} M ACh for CPO-I and CPO-III and 66.23×10^{-5} M ACh for CPO-II substrate containing buffers. The signal change at each pH value was evaluated using the expression $((I_o - I)/I_o)100$ in which I_o represents the fluorescence intensity response of the sensor systems in plain buffers of 2.5 mM phosphate (pH 6.5, 7.0, 7.5, 8.0, 8.2) and I represents the fluorescence intensity response of the sensor systems in the presence of AChE containing buffers (Fig. 3.). pH 7.0 was chosen as an appropriate measurement medium for CPO-II and CPO-III and pH 7.5 for CPO-I. In order to avoid other interfering pH dependent signal fluctuations, we recorded all of the spectra in phosphate buffered solutions.

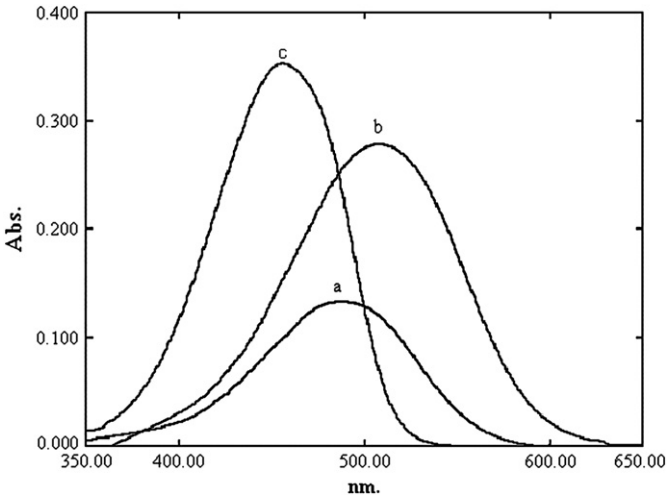


Fig. 1. Absorption spectra of (a) CPO-I ($\lambda_{max} = 488$ nm), (b) CPO-II ($\lambda_{max} = 509$ nm) and (c) CPO-III ($\lambda_{max} = 458$ nm) in PVC (2×10^{-6} mol CPO/kg PVC, 4 unit AChE activity).

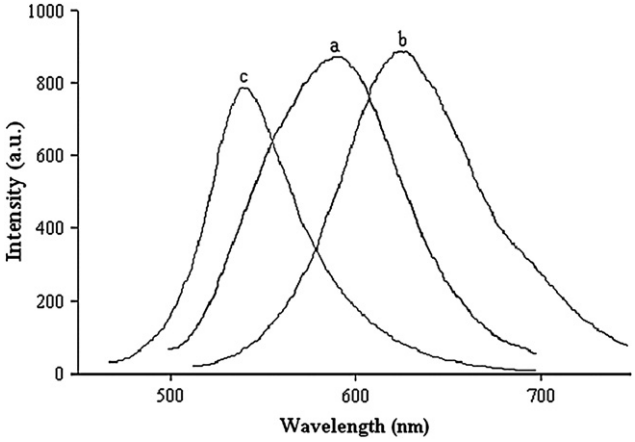


Fig. 2. Emission spectra of (a) CPO-III ($\lambda_{max} = 539$ nm), (b) CPO-I ($\lambda_{max} = 590$ nm) and (c) CPO-II ($\lambda_{max} = 625$ nm) in PVC (2×10^{-6} mol CPO/kg PVC CPO, 4 unit AChE activity).

3.2.2. Response time

The response time of the sensor membrane was investigated in 2.5 mM, pH 7.0 phosphate buffer for CPO-II and CPO-III and at pH 7.5 for CPO-I after the addition of 8.33×10^{-5} M ACh for CPO-I and CPO-III and 66.23×10^{-5} M ACh for CPO-II (Fig. 4). Maximum change in fluorescence intensity due to enzymatic hydrolysis of the substrate was observed after 4 min for all CPO derivatives; this remained constant over time. Thus, the optimum response time of the sensor systems was chosen as 4 min.

3.3. Characterization studies

Sensor slides respond to a proton resulting from enzymatic activity. The release of the acid anion decreases the pH of the bulk electrolyte solution which was converted to an optically measurable signal by the immobilized dye. The analytical signal corresponds to the rate of decrease in fluorescence intensity.

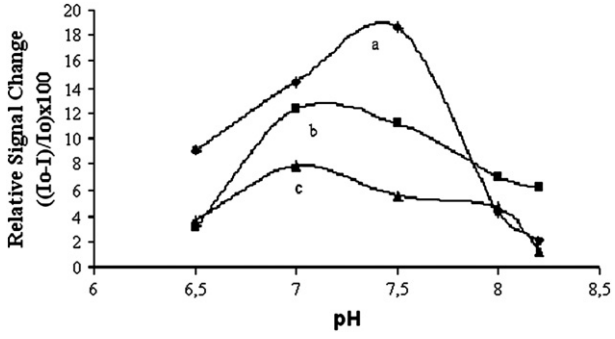


Fig. 3. pH dependence of the fluorescence intensity of (a) CPO-I, (b) CPO-II and (c) CPO-III in PVC (2×10^{-6} mol CPO/kg PVC, 4 unit AChE activity) in the pH range of 6.5–8.2, in 2.5 mM phosphate buffer; I_o refers to fluorescence intensity of CPO derivatives in plain buffers of 2.5 mM phosphate and I refers to buffers containing 8.33×10^{-5} M ACh for CPO-I and CPO-III and 66.23×10^{-5} M ACh for CPO-II.

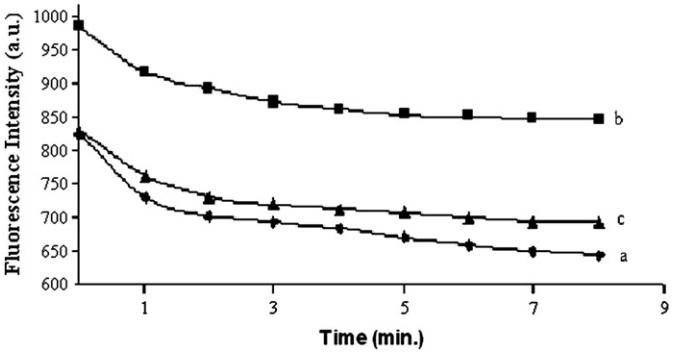


Fig. 4. Fluorescence intensity decrease of (a) CPO-III, (b) CPO-I and (c) CPO-II in PVC (2×10^{-6} mol CPO/kg PVC, 4 unit AChE activity) in 2.5 mM, pH 7.0 phosphate buffer for CPO-II and CPO-III, and pH 7.5 for CPO-I, after addition of 8.33×10^{-5} M ACh for CPO-I and CPO-III and 66.23×10^{-5} M ACh for CPO-II.

The analytical characteristics of CPO-based biosensing systems in terms of linear range, equation, limit of detection (LOD), etc. for ACh are summarized in Table 2.

The LOD values obtained reveal that the sensitivity of the sensor membranes of CPO-II was approximately 15–19-fold lower than that for the other two. This can be attributed to the electron deficiency of the possible locations of proton attack in the molecules caused by the nitro group located in the *para* position of phenyl.

As shown in Table 2, the linear range of the systems for the ACh substrate was found to be at the sub mM level, which displayed better sensitivity compared to previous work that utilised a fibre optic, based on immobilized AChE and thymol blue biosensor [27] and an optical sensor consisting of a chromoionophore doped sol–gel film interfaced with another sol–gel film immobilized with AChE [2]. An optical system based on ChE/liposome was found to be less sensitive towards to AChE substrate, ATChCl which was at the mM level [8].

3.3.1. Reproducibility and reversibility

The reproducibility and reversibility of the sensor compositions were tested by repeatedly exposing the PVC slides to Ach; the reagent phase was regenerated by 2.5 mM phosphate buffer. The drifts of the upper signal obtained for five cycles and the level of reproducibility achieved are given in Table 3. The RSD values achieved for the response of all three biosensors were very low indicating high reproducibility; the drift

Table 2
Analytical characteristics of CPO-I, CPO-II and CPO-III based biosensing systems in PVC matrix for ACh

CPO	Linear range (M)	Equation[a]	R^2	LOD[b] (M)
CPO-I	1.67×10^{-5}–33.22×10^{-5}	$y = 0.0138x + 1$	0.997	1.01×10^{-5}
CPO-II	3.30×10^{-4}–32.26×10^{-4}	$y = 0.0069x + 1$	0.982	1.91×10^{-4}
CPO-III	1.67×10^{-5}–13.32×10^{-5}	$y = 0.0118x + 1$	0.992	1.23×10^{-5}

[a] y and x show fluorescence intensity (a.u.) and concentration (M), respectively.
[b] LOD values were calculated by S/N ($n = 3$).

Table 3
The drifts of the upper signal obtained for the following five cycles and RSD values of CPO-I, CPO-II and CPO-III in PVC matrix to ACh[a]

CPO	Drifts (%)					RSD (%)
CPO-I	9.2	9.4	9.5	9.7	9.8	0.4
CPO-II	5.5	5.7	6.0	6.5	6.7	0.4
CPO-III	4.9	5.3	5.4	5.6	5.7	0.4

[a] ACh concentrations: 33.2×10^{-5} M, 32.3×10^{-4} M and 13.32×10^{-5} M for CPO-I, CPO-II and CPO-III.

values obtained reveal that the sensor slides were reversible in ACh measurement.

3.4. Sensor response for AChE inhibitor: donepezil

As already mentioned, donepezil treatment inhibits enzyme activity and so decreases the fluorescence intensity. Inhibition levels were found to be well correlated with donepezil concentration. Fig. 5 shows the emission spectra of CPO-I in PVC (2×10^{-6} mol CPO-I/kg PVC, 4 unit AChE activity) in 2.5 mM pH 7.5 phosphate buffer containing 33.22×10^{-5} M ACh in the concentration range of $7.99–443.86 \times 10^{-7}$ M donepezil.

The reproducibility and reversibility of sensor compositions based on CPO-I were tested by repeatedly exposing the sensor slides to a donepezil concentration of 443.86×10^{-7} M in 2.5 mM pH 7.5 phosphate buffer containing 33.22×10^{-5} M ACh. Each time the reagent phase was regenerated using phosphate buffer at pH 7.0. The relative standard deviation was calculated for the upper signal and was found to be 0.6% for five measurements.

The emission spectra of CPO-II in PVC matrix in 2.5 mM pH 7.0 phosphate buffer containing 32.26×10^{-4} M ACh in the concentration range of $7.99–412.43 \times 10^{-7}$ M donepezil is shown in Fig. 6.

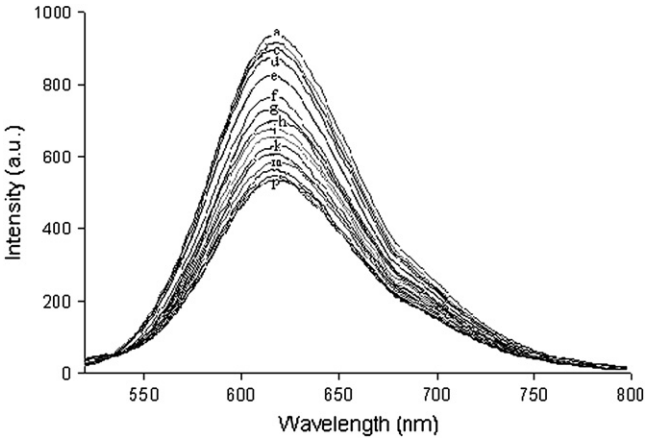


Fig. 6. Emission spectra of CPO-II in PVC (2×10^{-6} mol CPO-II/kg PVC, 4 unit AChE activity) in 2.5 mM pH 7.0 phosphate buffer containing 32.26×10^{-4} M ACh after exposure to different donepezil concentrations (a) 0, (b) 7.99, (c) 15.99, (d) 31.98, (e) 63.91, (f) 95.81, (g) 127.66, (h) 159.47, (i) 191.24, (j) 222.96, (k) 254.64, (l) 286.28, (m) 317.88, (n) 349.44, (o) 380.95 and (p) 412.41×10^{-7} M.

The reproducibility and reversibility of the sensor composition based on CPO-II were determined by repeatedly exposing the sensor slides to a donepezil concentration of 412.43×10^{-7} M in 2.5 mM pH 7.0 phosphate buffer containing 32.26×10^{-4} M ACh. Each time the reagent phase was regenerated using phosphate buffer at pH 7.0. The relative standard deviation was found to be 0.4% for five measurements. Fig. 7 shows the emission spectra of CPO-III in PVC in 2.5 mM pH 7.0 phosphate buffer containing 13.32×10^{-5} M ACh in the concentration range of $7.99–317.88 \times 10^{-7}$ M donepezil.

In terms of the reproducibility and reversibility of the CPO-III sensor composition, repeated exposure to a donepezil

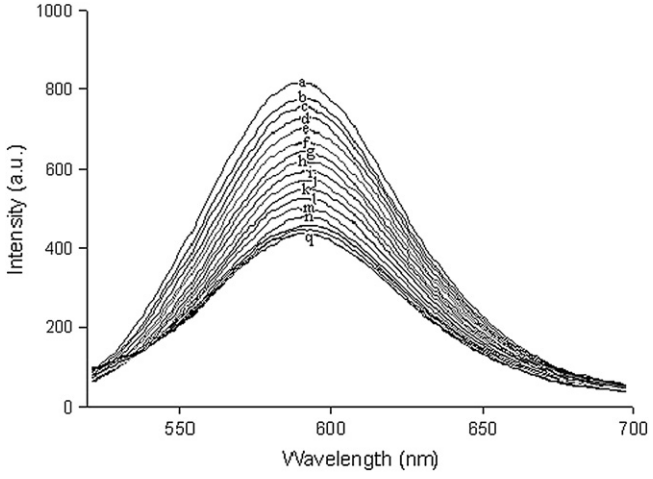


Fig. 5. Emission spectra of CPO-I in PVC (2×10^{-6} mol CPO-I/kg PVC, 4 unit AChE activity) in 2.5 mM pH 7.5 phosphate buffer containing 33.22×10^{-5} M ACh after exposure to different donepezil concentrations (a) 0, (b) 7.99, (c) 15.99, (d) 31.98, (e) 63.91, (f) 95.81, (g) 127.66, (h) 159.47, (i) 191.24, (j) 222.96, (k) 254.64, (l) 286.28, (m) 317.88, (n) 349.44, (o) 380.95, (p) 412.41 and (q) 443.86×10^{-7} M.

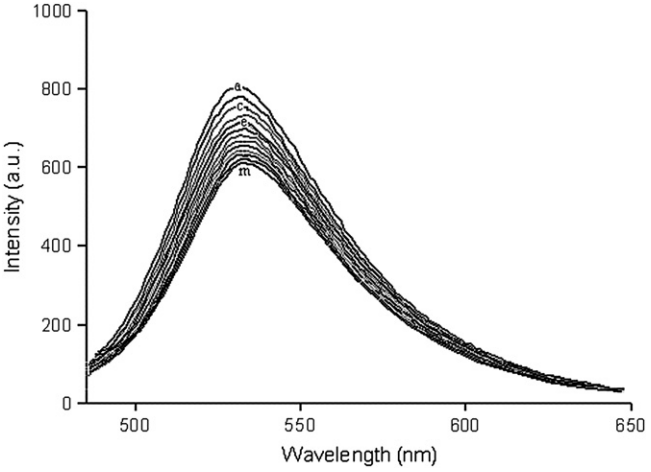


Fig. 7. Emission spectra of CPO-III in PVC (2×10^{-6} mol CPO-III/kg PVC, 4 unit AChE activity) in 2.5 mM pH 7.0 phosphate buffer containing 13.32×10^{-5} M ACh after exposure to different donepezil concentrations (a) 0, (b) 7.99, (c) 15.99, (d) 31.98, (e) 63.91, (f) 95.81, (g) 127.66, (h) 159.47, (i) 191.24, (j) 222.96, (k) 254.64, (l) 286.28, and (m) 317.88×10^{-7} M.

Table 4
Analytical characteristics of CPO-I, CPO-II and CPO-III in PVC matrix for donepezil (in 2.5 mM pH 7.5 phosphate buffer, ACh: 33.22×10^{-5} M, 32.26×10^{-4} M, and 13.32×10^{-5} M, for CPO-I, CPO-II and CPO-III, respectively)

CPO	Linear range (M)	Equation[a]	R^2	LOD[b] (M)
CPO-I	7.99×10^{-7}–443.86×10^{-7}	$y = 0.002x + 1$	0.990	3.48×10^{-7}
CPO-II	7.99×10^{-7}–412.43×10^{-7}	$y = 0.0019x + 1$	0.991	5.02×10^{-7}
CPO-III	7.99×10^{-7}–317.88×10^{-7}	$y = 0.0011x + 1$	0.992	5.92×10^{-7}

[a] y and x show fluorescence intensity (a.u.) and concentration (M), respectively.
[b] LOD values were calculated by S/N ($n = 3$).

concentration of 317.88×10^{-7} M in 2.5 mM pH 7.0 phosphate buffer containing 13.32×10^{-5} M Ach and regenerating the reagent phase using phosphate buffer at pH 7.0, revealed that the relative standard deviation was 0.8% for five measurements. Hence, all three sensor systems were fully reversible.

The analytical characteristics of CPO-based biosensing system for donepezil detection are summarized in Table 4.

According to the LOD and RSD values in Table 4, the developed biosensors were sensitive and reproducible to donepezil and there were no considerable differences in the responses of the CPs.

3.5. Sensor stability

Sensor stabilities were determined in plain buffers of 2.5 mM phosphate and in the presence of 8.33×10^{-5} M, 13.16×10^{-4} M and 8.33×10^{-5} M ACh containing buffers for CPO-I, CPO-II and CPO-III, respectively. The fluorescence intensity changes as a function of time for the same sensor film over 31 consecutive days of measurements are given in Table 5. The results obtained indicate that the stabilities of the sensor slides were satisfactory.

4. Conclusions

Determination of ACh and donepezil was accomplished by the use of newly synthesized CPO-I, CPO-II and CPO-III in plasticized PVC matrices. The sensor membranes were sensitive to ACh in the ranges 1.67–33.22×10^{-5} M, 3.30–32.26×10^{-4} M and 1.67–$13.32\ 10^{-5}$ M for an average exposure time of 4 min for CPO-I, CPO-II and CPO-III, respectively. The enzyme probes can be used for donepezil sensing in the concentration ranges 7.99–443.86×10^{-7} M, 7.99–412.43×10^{-7} M and 7.99–317.88×10^{-7} M for CPO-I, CPO-II and CPO-III, respectively. The CPO-III sensor membranes were more sensitive to ACh than CPO-I and CPO-II due to electron enrichment of the molecule caused by the *o*-tolyl group. The sensor systems were fully reversible and reproducible, showing that the fluorescent CPO derivatives offer use as alternative indicators for enzymatic neurotransmitter and donepezil sensing.

Table 5
The fluorescence intensity changes vs. time for the same sensor films for CPO-I, CPO-II and CPO-III

Time (days)	Fluorescence intensity					
	CPO-I		CPO-II		CPO-III	
	Plain buffer	ACh containing buffer	Plain buffer	ACh containing buffer	Plain buffer	ACh containing buffer
1	709	675	670	645	763	712
6	705	673	669	643	761	710
11	704	671	665	641	760	706
16	702	670	663	638	757	703
21	698	669	660	634	755	701
26	695	667	658	632	751	695
31	693	663	655	631	748	691

Acknowledgements

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