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PAPER

Determination of catecholamine in human serum by a fluorescent quenching method based on a water-soluble fluorescent conjugated polymer–enzyme hybrid system

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In this paper, a sensitive water-soluble fluorescent conjugated polymer biosensor for catecholamine (dopamine DA, adrenaline AD and norepinephrine NE) was developed. In the presence of horse radish peroxidase (HRP) and H_2O_2, catecholamine could be oxidized and the oxidation product of catecholamine could quench the photoluminescence (PL) intensity of poly(2,5-bis(3-sulfonatopropoxy)-1,4-phenylethynylenealt-1,4-poly(phenylene ethynylene)) ($PPESO_3$). The quenching PL intensity of $PPESO_3$ (I_0/I) was proportional to the concentration of DA, AD and NE in the concentration ranges of 5.0×10^{-7} to 1.4×10^{-4}, 5.0×10^{-6} to 5.0×10^{-4}, and 5.0×10^{-6} to 5.0×10^{-4} mol L^{-1}, respectively. The detection limit for DA, AD and NE was 1.4×10^{-7} mol L^{-1}, 1.0×10^{-6} and 1.0×10^{-6} mol L^{-1}, respectively. The $PPESO_3$–enzyme hybrid system based on the fluorescence quenching method was successfully applied for the determination of catecholamine in human serum samples with good accuracy and satisfactory recovery. The results were in good agreement with those provided by the HPLC-MS method.

Introduction

Biosensors are devices for detecting biochemical substances such as biological proteins, nucleotides and even tissues,[1–3] and are composed of an active sensing material with a signal transducer. Various biosensors, according different output signals, have been reported including electrochemical,[4] coulometric,[5] fluorescence,[6] spectrophotometric,[7–9] luminescent,[10–12] chromatographic,[13–17] and kinetic[18] procedures. Compared with other methods, optical biosensors based on absorbance and fluorescence detection are more advanced because of several advantages.[19,20] For example, the response mechanism of optical detection is very classic (widely known and agreed), simple, fast and reliable; the equipment is very common, easy to use and the reactions taking place in sample solutions are not disturbed because optical transducers do not change the component in the solutions. Recently, optical biosensors have been paid more attention.

Among fluorescence probes, more and more attention has been paid on conjugated polymers (CPs) owing to their superior signal amplification and superquenching properties. The amplification and superquenching properties of conjugated polymers result from the conjugated polymer backbone,[21] on which one single quencher molecule can cause effective and fast quenching. In view of the unique optical properties, conjugated polymers have been used as an optical platform in highly sensitive chemical and biological sensors to selectively detect metal ions, organic compounds, biological small molecules, enzymes, nucleic acids and proteins.[22–28]

Catecholamines which contain catechol (CA) or 3,4-dihydroxybenzene are part of the sympathetic nervous system. In the human body, the most abundant catecholamines are dopamine (DA), adrenaline (AD) and norepinephrine (NE). These catecholamines, which influence a variety of motivated behaviors, attention span, and neuronal plasticity, play a critical role in learning and memory.[29,30] In particular, DA, a catecholamine neurotransmitter, widely presents in the central nervous system where it modulates several aspects of the brain circuitry. A lack of DA in body fluids causes serious diseases such as anorexia, Parkinson's disease and schizophrenia.[31] In a recent study, the concentration of dopamine in serum and urine is related to the heart rate which reflects the pressure the people endured. The concentration of DA on the urine of diabetes and kidney disease patients is an important reference to the pathogenesis of hypertension. Nowadays, catecholamine-based pharmaceuticals are widely used in medicine. So it is extremely important to measure DA precisely and accurately. Yang and co-workers developed a method for the detection of catecholamine based on the fluorescence quenching of CdSe nanocrystals.[32] They also developed an aptamer-based colorimetric biosensor for DA using unmodified gold nanoparticles.[33] The tyrosinase–Fe_3O_4 nanoparticles–chitosan nanocomposite[34] and peroxidase-based method[35] were also used for detecting DA by Zhang's group and Poliakov *et al.*,

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respectively. Compared to quantum dots and metal nanoparticles, CPs have many advantages as biosensors for detecting various analytes in biosystems, such as they do not contain lead, cadmium and other heavy metals, which are harmful to the organism, and CPs have fluorescence amplification and superquenching properties. Up to now, biosensors based on the fluorescence quenching of CPs by catecholamine have not been reported and indeed they might have wide potential application in the development of catecholamine biosensors. Based on the superior signal amplification and superquenching properties of CPs and the high sensitivity and selectivity of enzymes, developing a facile and sensitive conjugated polymers–enzyme hybrid system for the detection of catecholamine could be an appealing alternative.

Poly(2,5-bis(3-sulfonatopropoxy)-1,4-phenylethynylenealt-1,4-poly(phenylene ethynylene)) ($PPESO_3$) has good water-solubility owing to its sulfonic acid group and has drawn great attention as an optical transducer in highly sensitive bio- and chemo-sensors. We have designed an HRP–H_2O_2–$PPESO_3$ system to detect both hydroquinone and hydrogen peroxide with satisfactory results.[36] In this study, we found that the oxidation product of catecholamine could quench the fluorescence of the water-soluble conjugated polymer $PPESO_3$ and that the quenching PL intensity ratio (I_0/I) was proportional to the concentration of catecholamine. Thus we developed a water-soluble fluorescent conjugated polymer–enzyme hybrid system which could be used as a sensitive fluorescence biosensor for the detection of DA, AD and NE (Scheme 1). The established biosensor method was successfully applied for the determination of catecholamine in human serum samples with good accuracy and satisfactory recovery. This method provides a new biology application for water-soluble fluorescent conjugated polymer.

Experimental

Chemicals

Tetrakis(triphenylphosphine)palladium ($(PPh_3)_4Pd$) was obtained from Hangzhou Kaida Metal Catalyst & Compounds Co. Ltd. 1,4-Diethynylbenzene and 1,3-propanesultone were purchased form Aldrich Chemical Co. and J&K Chemica respectively. 2,5-Diiodohydroquinone and dioxane were obtained form Tianjin Guangfu Institute of elaborate chemical industry. Horseradish peroxidase (HRP) was obtained from Sino-American Biotechnology Co. Ltd. Catechol was purchased from Tianjin Guangfu Institute of elaborate chemical industry. Dopamine hydrochloride injection (2 ml: 20 mg), adrenaline hydrochloride injection (1 ml: 1 mg) and noradrenaline bitartrate injection (1 ml: 2 mg) from Shanghai Harvest pharmaceutical Co. Ltd were used as received. All the other chemicals, including CuI, Na_2HPO_4, NaH_2PO_4, H_2O_2, methanol, acetone, diethyl ether and DMF were obtained from Beijing Chemical Co. Ltd. Stock solutions of 0.1 mol L^{-1} H_2O_2 and HRP were freshly prepared daily. All chemicals used were of analytical reagent grade without further purification. The water used in all experiments had a resistivity of 18 MΩ/cm. All the water used in the experiments was de-aerated by purging with N_2 for 30 min.

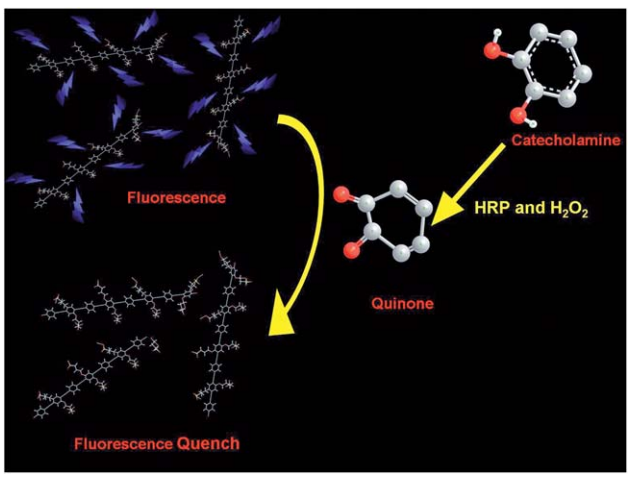
Instrumentation

All fluorescence measurements were carried out in a 1 cm path-length quartz cuvette at ambient temperature with a Shimadzu RF-5301 spectrometer. In all optical experiments, all the FL detections were under the same conditions: the excitation wavelength was 400 nm and the fluorescence intensity referred to the maximum emission of $PPESO_3$ which was at 520 nm. The slit widths of the excitation and emission were both 5 nm.

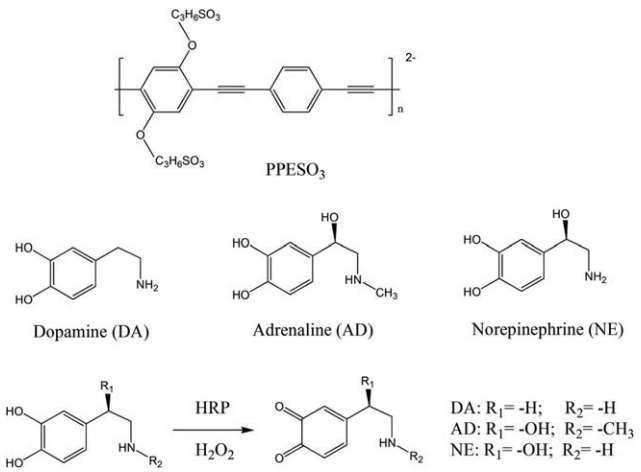
For the HPLC-MS method, an HPLC-MS/MS system with an Agilent 1100 liquid chromatograph (Palo Alto, CA, USA) and Q-Trap mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Canada) equipped with an electro-spray ionization (ESI) source and an automatic ten-port switching valve (V 2) was used.

Experimental method

The synthesis of $PPESO_3$ (see Scheme 2 for the chemical structure) was according to the previous paper.[37] For the assay of dopamine, certain amounts of dopamine stock solution were added to cuvettes and diluted to 1 mL with PBS (pH 7.4) buffer,



Scheme 1 Schematic illustration of the fluorescence quenching of HRP–H_2O_2–$PPESO_3$ system by catecholamine.



Scheme 2 The molecular structures of $PPESO_3$, DA, AD, NE and the scheme of DA, AD and NE oxidation catalyzed by HRP and H_2O_2.

thus a series of dopamine solutions with different concentrations were obtained. 1 mL of 2.0 μmol L^{-1} $PPESO_3$ in PBS (pH 7.4) solution and 1 mL of DA solution with a series of concentrations (see the captions to Fig. 1 and Fig. 5) were added to a quartz cuvette, followed by the addition of 2.0 μg mL^{-1} HRP and 2.0 mmol L^{-1} H_2O_2. The resulting solution (2.0 mL) was shaken evenly and kept at room temperature for 20 min before recording the spectral information by spectrofluorophotometer. The method of detecting CA, AD and NE was the same as that of DA.

For serum samples detection, drug-free human blood samples were collected from healthy volunteer and hypertension patient treated at the Hospital of Changchun China Japan Union Hospital. All experiments were performed in compliance with the relevant laws and institutional guidelines, and written informed consent was obtained for all samples obtained from human subjects. All the blood samples were obtained by venipuncture and centrifuged at 10 000rpm for 10 min after standing for 2 h at room temperature. When used, the serum sample was thawed and deproteinized by adding 300 μL of acetonitrile to 200 μL sample. After vigorously shaking for 2 min, the mixture was centrifuged at 10 000 rpm for 8 min at 4 °C. Aliquots of the supernatant were used for the derivatization as described above. The samples were diluted 100 times with 0.02 mol L^{-1} PBS (pH 7.4). To the diluted serum samples were added different concentrations of DA, AD or NE separately (10 μmol L^{-1}, 100 μmol L^{-1}) to prepare the spiked samples.

Results and discussion

Quenching by DA in the presence of HRP and H_2O_2

Our previous study indicates that the enzymatic reaction product of hydroquinone–quinone can efficiently quench the photoluminescence (PL) intensity of $PPESO_3$.[36] Considering that DA is a catecholamine that contains a dihydroxybenzene group, we supposed that the enzymatic reaction product of catecholamine may also quench the PL intensity of $PPESO_3$. As a model system of the hydroquinone, the quenching effect of DA with the HRP-catalyzed oxidation by H_2O_2 was investigated. $PPESO_3$ aqueous solution mixed with H_2O_2, DA and HRP separately and the mixtures of two or three were investigated and the results were shown in Fig. 1. It can be seen from Fig. 1 that when $PPESO_3$ mixed with H_2O_2, DA or HRP separately, the decrease of the PL intensity of $PPESO_3$ was no more than 10%, which indicates that none of the three agents could act as a PL quencher solely. The PL quenching of $PPESO_3$ could not be observed even when both H_2O_2 and DA exist, which implies that no quencher (which could accept the electron transferred from $PPESO_3$) was produced. Then upon sequential addition of DA, H_2O_2 and HRP into the $PPESO_3$ aqueous solution, remarkable PL quenching (nearly 74%) can be observed. These results showed that a spot of DA can be oxidized by H_2O_2 in the presence of HRP and the oxidation product of DA as a effective quencher can effectively quench the PL intensity of $PPESO_3$ (Scheme 1).

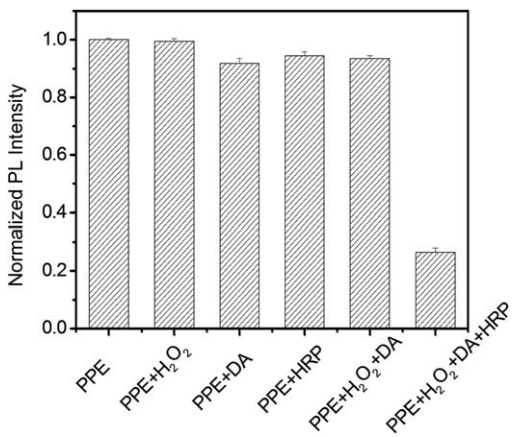


Fig. 1 The effect of HRP as catalyst on the oxidation of DA in the absence/presence of HRP/H_2O_2 in 1.0 μmol L^{-1} $PPESO_3$. 1.0 μmol L^{-1} $PPESO_3$ without any addition; with the addition of 2.0 mmol L^{-1} H_2O_2; with the addition of 6.5 × 10^{-5} mol L^{-1} DA; with the addition of 6.5 × 10^{-5} mol L^{-1} DA and 2.0 mmol L^{-1} H_2O_2; with the addition of 6.5 × 10^{-5} mol L^{-1} DA, 2.0 mmol L^{-1} H_2O_2 and 2.0 μg mL^{-1} HRP.

Effect of incubation time on DA detection

For the kinetic study of the quenching effects, the quenching effect of DA on the $PPESO_3$ fluorescence intensity was investigated in the present work. DA, H_2O_2 and HRP were added into $PPESO_3$ aqueous solution and the mixture underwent a fluorescence experiment immediately. Fig. 2 shows the quenching effects of 6.5 × 10^{-5}mol L^{-1} DA on the $PPESO_3$ PL intensity with different incubation times. It can be seen that the oxidation product of DA presents efficient quenching on $PPESO_3$ PL intensity, the quenching effect increased with the increase of incubation time and the PL intensity remains unchanged after 20 min. The results proved that the catalyzed oxidation process of DA finished in 20 min. In the following study, the incubation time of 20 min was adopted.

Effect of pH on DA detection

The effect of the pH on DA detection was studied in this work. From Fig. 3, it can be seen that the fluorescence intensity of $PPESO_3$ at 520 nm is nearly unchanged in the pH range of 6.0 to 10.0, whereas for the DA–HRP–H_2O_2–$PPESO_3$ system, the fluorescence intensity of $PPESO_3$ at pH 7.4 was markedly higher than that under other pH conditions, which indicates that a pH

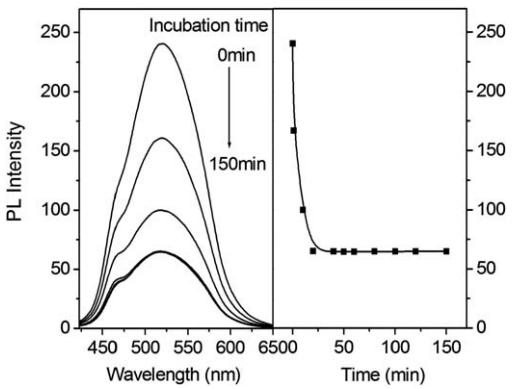


Fig. 2 The relationship between the PL intensity of HRP–H_2O_2–$PPESO_3$–DA system and the incubation time.

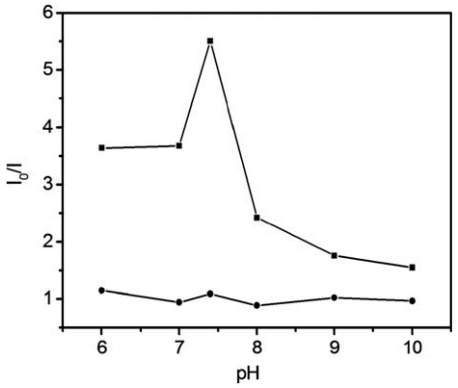


Fig. 3 Effect of pH on the PL intensity of $PPESO_3$ (●) and HRP–H_2O_2–$PPESO_3$–DA (■) system at 520 nm.

value approaching that of physiological conditions is good for the HRP catalysis reaction. So a pH of 7.4 was chosen in this study to provide an optimal physiological condition for DA determination.

Effect of HRP and H_2O_2 on DA detection

The influence of the concentrations of HRP and H_2O_2 on DA detection in the HRP–H_2O_2–$PPESO_3$ system is illustrated by Fig. 4a. The effect of HRP concentration on the $PPESO_3$ PL intensity was investigated at DA concentrations of 6.5×10^{-7} mol L^{-1} (▲), 6.5×10^{-6} mol L^{-1} (●) and 6.5×10^{-5} mol L^{-1} (■). From Fig. 4a, It can be seen that the PL quenching extent was enhanced with increasing the concentration of DA. The PL intensity of $PPESO_3$ decreased 13.97%, 36.37% and 71.36%, when the concentrations of DA increased from 6.5×10^{-7} to 6.5×10^{-5} mol L^{-1}. On the other hand, the PL intensity of $PPESO_3$ decreased dramatically after adding of HRP and maintained the same when the concentration of HRP increased. These results indicate that the quantity of HRP did not depend on the concentration of DA. Thus, 2.0 μg mL^{-1} HRP was adopted in following experiments. The effect of concentration of H_2O_2 on DA detection was also studied, and the results were shown in Fig. 4b. From Fig. 4b we found that when the concentration of H_2O_2 reached 2.0 mmol L^{-1}, the fluorescence intensity of $PPESO_3$ remained nearly unchanged with further increase in concentration of H_2O_2, which indicated that 2.0 mmol L^{-1} of H_2O_2 can oxidize DA adequately in this system.

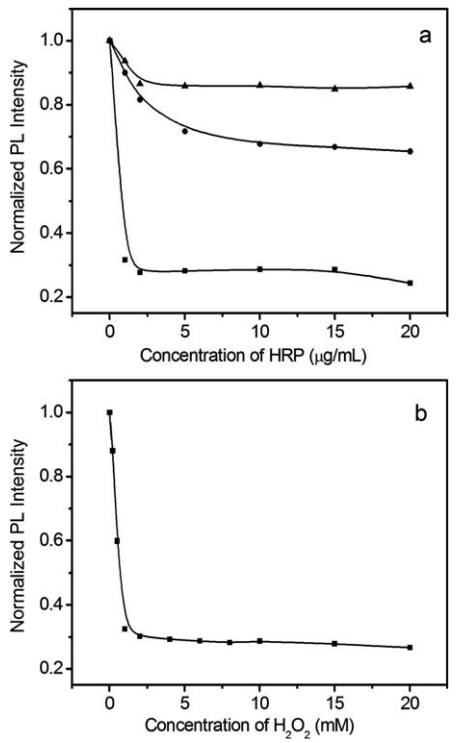


Fig. 4 (a) Effect of HRP concentration on the fluorescence quenching of HRP–H_2O_2–$PPESO_3$ system by DA. (b) Effect of H_2O_2 concentration on the fluorescence quenching of HRP–H_2O_2–$PPESO_3$ system by DA.

Detection of DA *via* the HRP–H_2O_2–$PPESO_3$ system

The quenching effect of DA on the PL intensity of $PPESO_3$ in the presence of HRP and H_2O_2 was further investigated under the optimized conditions. Fig. 5 shows the fluorescence spectra of $PPESO_3$ in the presence of 2.0 mmol L^{-1} H_2O_2, 2.0 μg mL^{-1} HRP and a series of different DA concentrations. As shown in Fig. 5, the PL intensity of $PPESO_3$ reduced gradually upon increasing the concentration of DA and there are no obvious changes of spectral shape on the fluorescence emission intensity. The inset of Fig. 5 indicated that the PL intensity ratio I_0/I of $PPESO_3$ (I_0 and I are the fluorescence intensity of $PPESO_3$–HRP–H_2O_2 system in the absence and presence of the DA, respectively) shows a good linear relationship in the range of 5×10^{-7} to 1.4×10^{-4} mol L^{-1} with a correlation coefficient $R^2 = 0.998$. The linear regression equation is $I_0/I = 1.130 + 3.69 \times 10^4 C_{DA}$ (mol L^{-1}) and the detection limit (3σ) for DA was found to be 1.4×10^{-7} mol L^{-1}. AD and NE with a similar structure to DA (Scheme 2) have the analogous chemical property. Thus they can also be quantified with the HRP–H_2O_2–$PPESO_3$ system (Fig. 6). The linear regression equation and the detection limit of AD and NE were shown in Table 1. From Table 1, it can be seen that the linear regression equations of phenol, catechol, AD and NE are

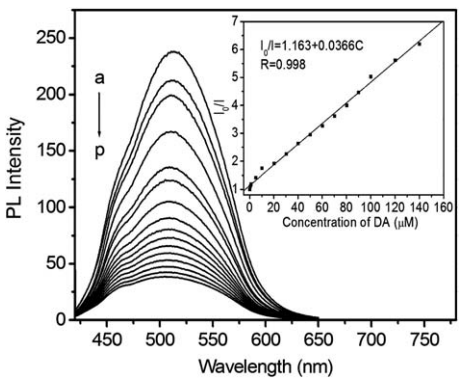


Fig. 5 The effect of DA concentration on the PL intensity of 1.0 μmol L^{-1} $PPESO_3$ in the presence of 2.0 mmol L^{-1} H_2O_2 and 2.0 μg mL^{-1} HRP; (a–p) represent the concentrations of DA of 0, 0.5, 1.0, 5.0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140 μmol L^{-1}, respectively. The inset shows the linear relationship between I_0/I and the concentration of DA.

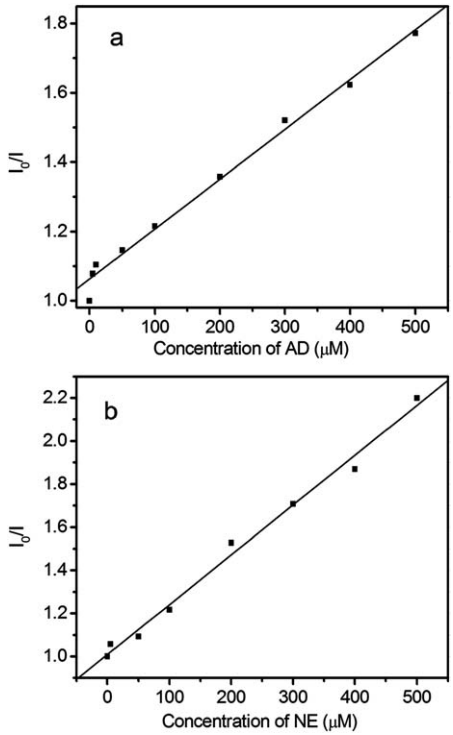


Fig. 6 The linear relationship between I_0/I and the concentration of AD and NE.

$I_0/I = 1.059 + 6.94 \times 10^4 C_{phenol}$ (mol L^{-1}); $I_0/I = 0.953 + 1.70 \times 10^4 C_{catechol}$ (mol L^{-1}); $I_0/I = 1.063 + 1.44 \times 10^3 C_{AD}$ (mol L^{-1}) and $I_0/I = 1.000 + 2.31 \times 10^3 C_{NE}$ (mol L^{-1}), respectively. The detection limits (3σ) of phenol, catechol, AD and NE are 6.5×10^{-8}, 8.0×10^{-8}, 1.0×10^{-6} and 1.0×10^{-6} mol L^{-1}, respectively. These results show that the fluorescence quenching method based on the $PPESO_3$–enzyme hybrid system can be applied for the determination of catecholamines with high sensitivity and over a wide linear range.

Interference study

The detection of selectivity of the $PPESO_3$–HRP–H_2O_2 system for DA was further evaluated with various substances added. The interference study was carried out at a DA concentration of 1×10^{-5} mol L^{-1} with various coexistence substrates added. The tolerance of each coexistence substrate was taken as the highest concentration yielding a relative error less than ±5%. We choose some coexistence substrates which are in normal existence in human serum. As it is shown in Table 2, the results indicted that the tolerable concentration ratio of coexisting substances to 10^{-5} mol L^{-1} DA was over 300-fold for Na^+, 500-fold for K^+,1000-fold for Cl^-, 50-fold for Ca^{2+}, Mg^{2+} and Zn^{2+}, and 20-fold for cysteine and glycine.

Table 2 The interference of coexisting substances on the determination of 1×10^{-5} mol L^{-1} DA

Coexisting substances	Coexisting times	RSD (%, $n = 3$)
Na^+	300	3.0
K^+	500	4.4
Cl^-	1000	4.6
Ca^{2+}	50	−3.8
Mg^{2+}	50	−2.6
Zn^{2+}	50	−4.9
Cysteine	20	−1.12
Glycine	20	−0.86

Real samples detection

In order to evaluate the feasibility of the proposed method in real samples detection, the developed quenching method was applied to the determination of DA, AD, and NE in two human serum samples. The fluorescence method proposed in this paper was compared with the HPLC-MS method. The results were listed in Table 3. From Table 3, it can be seen that the results obtained by the $PPESO_3$–HRP–H_2O_2-based fluorescence quenching method were in good agreement with those provided by the HPLC-MS method. These results demonstrate that this new proposed fluorescence method can be applied in practical sample analysis, and that the results were satisfactory.

Table 3 Determination of DA, AD and NE in human serum samples by the proposed method and HPLC-MS method ($n = 3$)

		Found/μM		
Analyte	Added/μM	Proposed method	HPLC-MS	Recovery (%)
DA	10	10.35	10.14	103.5
	100	106.90	102.47	106.9
AD	10	9.63	9.85	96.3
	100	104.55	102.92	104.6
NE	10	10.76	10.08	107.6
	100	108.90	103.39	108.9

Table 1 Linear regression equation and detection limit of the compounds studied

Analytes	Linear regression equation	R^2	Detection limit/ mol L^{-1}	Concentration range/μmol L^{-1}
Phenol	$I_0/I = 1.059 + 6.94 \times 10^4 C$	0.997	6.5×10^{-8}	0.1∼20
Catechol	$I_0/I = 0.953 + 1.70 \times 10^4 C$	0.997	8.0×10^{-8}	0.1∼330
Dopamine (DA)	$I_0/I = 1.130 + 3.69 \times 10^4 C$	0.998	1.4×10^{-7}	0.5∼140
Epinephrine (AD)	$I_0/I = 1.063 + 1.44 \times 10^3 C$	0.995	1.0×10^{-6}	5∼500
Norepinephrine (NE)	$I_0/I = 1.000 + 2.31 \times 10^3 C$	0.996	1.0×10^{-6}	5∼500

Conclusions

In this paper, we found that oxidation product of catecholamines could quench the fluorescence of water-soluble conjugated polymer $PPESO_3$ and that the quenching PL intensity ratio (I_0/I) was proportional to the concentration of catecholamine. Thus we developed an HRP–H_2O_2–$PPESO_3$ system which could be used as a sensitive fluorescence biosensor for the detection of catechol, dopamine, adrenaline and norepinephrine. The quenching experimental parameters for DA were optimized and good selectivity and sensitivity were acquired for DA by this method. For real sample detection, we assayed DA, AD and NE in human serum samples. The accuracy of the results in the determination of DA, AD and NE in human serum samples obtained by the proposed fluorescence method was confirmed by using HPLC-MS as an alternative technique. The proposed method can be used to monitor DA in serum which may be used in the determination of catecholamines in different pharmaceutical formulations, drug delivery and drug release in the living body or cell in future investigations.

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