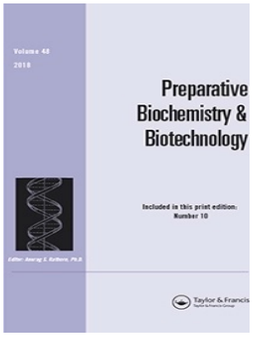


Preparative Biochemistry & Biotechnology

ISSN: 1082-6068 (Print) 1532-2297 (Online) Journal homepage: https://www.tandfonline.com/loi/lpbb20

Development of choline biosensor using toluidine blue O as mediator

Rüya Baskin, Elif (Aynaci) Koyuncu, Halit Arslan & Fatma Arslan

To cite this article: Rüya Baskin, Elif (Aynaci) Koyuncu, Halit Arslan & Fatma Arslan (2019): Development of choline biosensor using toluidine blue O as mediator, Preparative Biochemistry & Biotechnology, DOI: 10.1080/10826068.2019.1687518

To link to this article: https://doi.org/10.1080/10826068.2019.1687518

Published online: 09 Nov 2019.

PREPARATIVE BIOCHEMISTRY & BIOTECHNOLOGY
https://doi.org/10.1080/10826068.2019.1687518

Taylor & Francis
Taylor & Francis Group

Development of choline biosensor using toluidine blue O as mediator

Rüya Baskin[a], Elif (Aynaci) Koyuncu[a,b], Halit Arslan[c], and Fatma Arslan[c]

[a]Department of Chemistry, Institute of Sciences, Gazi University, Ankara, Turkey; [b]Department of Chemistry, Faculty of Engineering and Natural Sciences, İstanbul Medeniyet University, Istanbul, Turkey; [c]Department of Chemistry, Faculty of Sciences, Gazi University, Ankara, Turkey

ABSTRACT

A new choline oxidase (ChO) and toluidine blue O (TBO) based amperometric choline biosensor was reported in this article. An amperometric choline biosensor with immobilization of TBO (as a mediator), ChO onto polypyrrole–polyvinylsulphonate (PPy–PVS) film was accomplished on the surface of a platinum electrode. ChO was immobilized on PPy–PVS film by cross-linking with glutaraldehyde (GA). TBO was used as the mediator. Choline is oxidized to betaine and hydrogen peroxide in an oxygenated environment by ChO. Mediator reduced by reaction with hydrogen peroxide. The amperometric response was based upon the electrocatalytic properties of TBO. Optimum pH and temperature values were 7.0 and 30 °C, respectively. There was linearity between 1.0×10^{-8} and 2.0×10^{-8} M ($R^2 = 0.9805$). The detection limit of the biosensor was 1.0×10^{-9} M and response time of the biosensor was 200 s. The storage stability and reproducibility of the biosensor were also investigated. Interfering effect of several interferants such as ascorbic acid, uric acid, alanine, dopamine, paracetamol, cysteine, and glucose on the choline biosensor was examined. The developed biosensor was tested in determinations of content in a synthetic blood sample.

KEYWORDS

Biosensor; choline; polypyrrole; toluidine blue O

Introduction

Choline, which is an amino alcohol, has an important role in human organism. It is a significant food essential with some physiological goals.[1] Choline plays a remarkable role in the synthesis of phospholipids such as phosphatidylcholine and sphingomyelin, which constitute the cell membrane structure and the transport of excess triglycerides in the liver.[2] It is also an important source of betaine, the metabolite, and methyl group donor required for homocysteine metabolism.[3] Moreover, it is the precursor of acetylcholine, a neurotransmitter associated with cholinergic memory and muscle control. Studies have shown that the incidence of Alzheimer's disease is high in the absence of choline.[4] For this reason, it is essential to determine the presence of choline, especially for the diagnosis of neurodegenerative diseases such as Alzheimer's and Parkinson's.[5]

Beef, lamb and chicken liver, meat, eggs, spinach, cabbage, cauliflower, green beans, and human breast milk contain high amounts of choline. This substance is a component of biological fluids such as blood and amniotic fluid. In human plasma, fasting choline concentration was reported to be in the interval of 7–20 μM or 10 μM on average.[6]

Choline is a product formed by the hydrolysis of acetylcholine with the acetylcholine esterase enzyme.[7] While choline oxidase (ChO) oxidizes choline to betaine, oxygen is reduced to H_2O_2 as a result of this reaction (Fig. 1).

Choline can be identified in the brain tissue, serum, foods, and pesticides. Chromatographic (gas chromatography-mass spectrum, liquid chromatography, and ion chromatography) or spectrophotometric[8–11] and electrochemical (amperometric, potentiometric, and conductometric) methods are used to determine the amount of the choline.[12–15] Electrochemical methods are fast, sensitive, reproducible, efficient, and highly selective. Other methods have disadvantages such as being expensive and time-consuming. With electrochemical based biosensors, it is possible to make cheaper, faster, and sensitive determinations to low concentrations and highly selective substances. There are studies in the literature about electrochemical based choline biosensors.[7,16] Most of the amperometric cholinergic biosensors are based on direct oxidation of H_2O_2, which is enzymatically reactive, to anodic potential.[16–18] Species like ascorbic acid, uric acid, and paracetamol, that are electrochemically active in biological fluids, have interference effects at these potentials.[16–20] This problem can be avoided by reducing the working potential using mediators.[21,22]

Literature studies of choline biosensors using mediators such as Prussian blue and methylene blue are available.[7,22] However, a choline biosensor study using TBO as a mediator was not found. For this purpose in our work, the use of TBO has also been given the advantage of reducing the interference effects of the substances that can be interfered with by reducing the working potential.

CONTACT Fatma Arslan fatma@gazi.edu.tr Faculty of Sciences, Gazi University, Ankara, Turkey.
Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/lpbb.

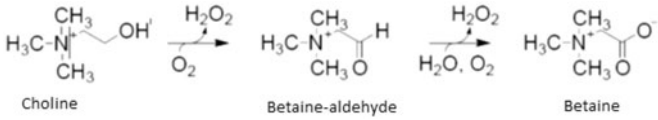


Figure 1. The reaction catalyzed by ChO.

In our work, we prepared a new amperometric biosensor sensitive to the choline. For this purpose, platinum/polypyrrole–polyvinylsulphonate (Pt/PPy–PVS) was prepared by electropolymerization. Subsequently, immobilization of the ChO in the presence of a mediator (TBO) was achieved by cross-linking to obtain Pt/PPy–PVS-ChO (TBO) enzyme. The choline determination was carried out on the basis of the oxidation of TBO at −0.23 V. The enhanced biosensor was examined in synthetic blood samples.

Materials and methods

Devices and chemicals

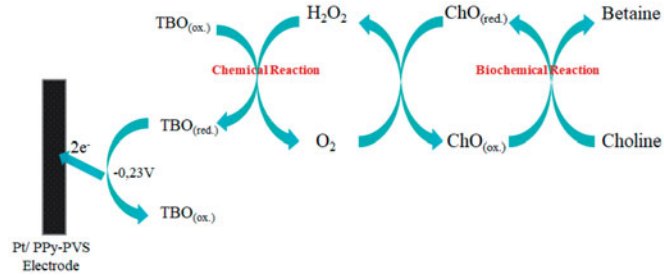
Electrochemical studies were made on a CH Instrument 1230B using a three-electrode cell. The working, auxiliary, and reference electrodes were Pt plate (0.5 cm^2), Pt wire, and Ag/AgCl electrode (3.0 M KCl), respectively. The pH measurements were done with ORION model 720 A pH/ion meter. Grant GD 120 thermostat was used for temperature control. ChO (E.C. 1.1.3.17, isolated from Alcaligenes species, the activity of 12.03 U) was provided from Sigma. Choline chloride was provided from Sigma. Pyrrole was procurement by Fluke and sodium polyvinyl sulfonate (PVS) by Aldrich. Chemicals except these two were provided from Sigma and solutions were prepared with pure water.

Deposition of PPy–PVS film on Pt

Platinum plate was cleaned mechanically and chemically before coating.[23] Then it was coated with PPy in PVS medium[24]. During the coating, polypyrrole was deposited on the electrode surface by electropolymerization of pyrrole. The coating process was done as follows; cleaned Pt electrode, auxiliary, and the reference electrode were dipped in a 10 mL solution of 0.1 M pyrrole and 2.5 mL (25%) of sodium polyvinylsulphonate. Argon gas was passed through the solution for 10 min to remove the oxygen. One cycle was obtained at a scanning speed of 50.0 mV/s between −1.0 and 2.0 V with a cyclic voltammetry (CV) technique (vs. Ag/AgCl electrode (3.0 M KCl)).[25] After coating, PPy–PVS film electrode was lavaged with pure water to remove unreacted pyrrole.

Fabrication of choline biosensor

After electropolymerization, 50.0 μL TBO (1.0×10^{-3} M) was dropped on the Pt/PPy–PVS film. The mix composed of 50.0 μL of ChO (1.203 U/mL), 1.0 mg of BSA, 50 μL of 0.1 M phosphate buffer at pH of 7.0, 45.0 μL of 2.5% glutaraldehyde (GA) was poured on PPy–PVS film. Then, film was dehydrated at 25 °C and cleaned several times with buffer solution. The prepared biosensor was kept at 4 °C in buffer, when not used.



Scheme 1. Reaction scheme for the choline determination.

Electrochemical studies

Choline detection was made by oxidation of TBO (red) at −0.23 V (vs. Ag/AgCl electrode (3 M KCl)).

$$\text{Choline} + 2O_2 \xrightarrow{\text{ChO}} \text{Betaine} + 2\,H_2O_2$$
$$2\,H_2O_2 + \text{TBO}_{\text{OX.}} \rightarrow 2O_2 + \text{TBO}_{\text{red.}}$$
$$\text{TBO}_{\text{red.}} \xrightarrow{-0.23\ \text{V}} \text{TBO}_{\text{OX.}}$$

The prepared biosensor was placed into 0.1 M phosphate buffer (pH 7.0). The solution composed 0.1 M KCl as an electrolyte solution. Biosensor was equilibrated at −0.23 V and constant current (i_a) was noted. Choline was put into the cell and current (i_b) received at −0.23 V was also noted. The graphic of current value ($\Delta i = i_b - i_a$) was drawn against the substrate concentration.

Results and discussion

As a result of this study, a novel amperometric choline biosensor based on ChO and toluidine blue O (TBO) was suggested. TBO has been chosen as an electron transfer mediator. Choline determination was made by the oxidation of $TBO_{(red)}$ at −0.23 V. Reaction Scheme 1 about detection of choline is indicated below.

The reaction of the choline with the ChO enzyme in the oxygen medium results in the formation of betaine and hydrogen peroxide. Hydrogen peroxide enters the chemical reaction with the mediator and the mediator is reduced. At the reduced mediator −0.23 V, anodic currents proportional to the concentration of the choline are obtained because of the oxidation at the electrode surface. This forms the working principle of a biosensor.

The detection of working potential

Pt/PPy–PVS electrodes were prepared, then cyclic voltammetry (CV) was performed between 0.3 V and (−0.4 V) using different concentrations (1.0×10^{-3} to 1.0×10^{-2} M) of TBO for the definition of the TBO's oxidation peak. It was observed at −0.23 V (Fig. 2). −0.23 V was taken as working potential for the following experiments.

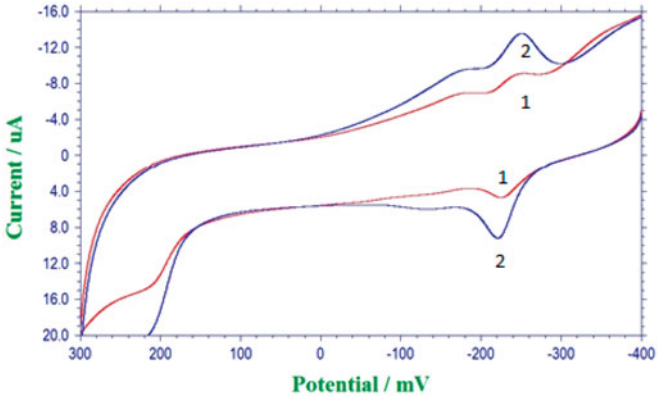


Figure 2. The cyclic voltammogram of the Pt/PPy–PVS electrode in 0.1 M phosphate buffer pH 7.0. (1) 1.0×10^{-3} M TBO (2) 1.0×10^{-2} M TBO.

Influence of glutaraldehyde amount on reply of biosensor

GA ($CHO–CH_2CH_2CH_2–CHO$) is an often utilized crosslinking agent. It takes reaction with enzyme molecules' amine groups. GA does not react with the enzyme surface's amine groups only but also can spread into enzyme molecules and coupling to internal amine groups.[25] So, the active site of the enzyme is devastated by excessive binding. It is important to investigate GA amount for performance of the sensor.

Different percentages of GA (2.5% (*v/v*)) solutions (0.33–0.94% (*v/v*)) were utilized for ChO immobilization. Figure 3 showed the following outcomes; activity of the crosslinking system raised when the amount of GA raised 0.33–0.78%. However, when GA amount increased to 0.94%, activity decreasing was observed. Therefore, 0.78% GA was determined as appropriate GA amount. Because, GA concentration of 0.33% was not sufficient, 0.94% was a lot more and resulted as loss in enzyme activity.

Influence of pH

The pH effect on the choline biosensor's response was observed in the pH range 6.0–10.0. Phosphate and glycine buffers at different pHs were used for this purpose. A fixed choline concentration of 5.0×10^{-5} mM was used at measurements. Highest response was seen for immobilized enzyme at pH 7.0 (Fig. 4). There were choline biosensor studies whose pH values varied than 7.0 in the references (7.4, 8.0).[21,26] Because the polymer and immobilization techniques used were divergent.

Influence of temperature

Temperature's effect on the reply of the biosensor was investigated among 20 °C and 50 °C at pH 7.0 utilization fixed choline concentration of 5.0×10^{-5} mM. Maximum electrode reply was observed at 30 °C for immobilized enzyme (Fig. 5).

In literature, different temperatures were used for choline biosensor (35, 25 °C).[27,28] The experiment was performed at 25 °C for practical measurements. Thus, room temperature 25 °C was selected as the working temperature in future studies.

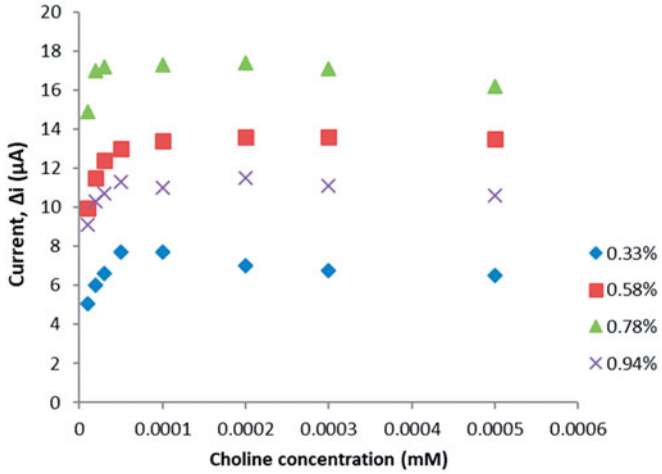


Figure 3. The effect of GA amounts on the response of the biosensor (25 °C).

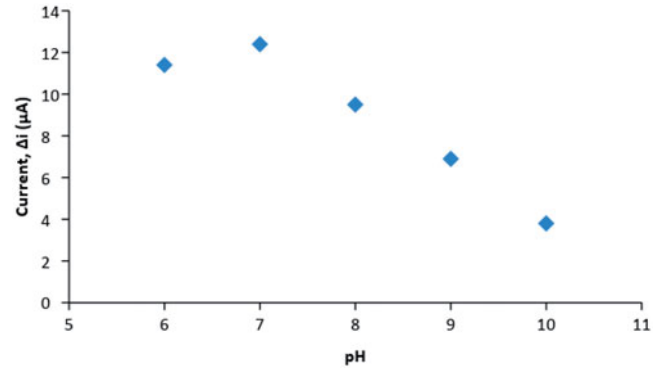


Figure 4. The effect of pH on the response of the biosensor (at pH 7.0, 5.0×10^{-5} mM choline at −0.23 V operating potential).

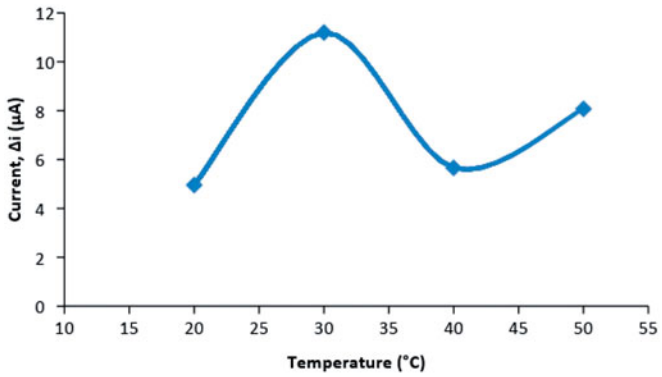


Figure 5. The effect of temperature on the response of the biosensor (at pH 7.0, 5.0×10^{-5} mM choline at −0.23 V operating potential).

Influence of substrate concentration on biosensor's reply and calibration curves

Influence of substrate concentration on biosensor's reply was investigated for solutions including 1.0×10^{-5} to 3.5×10^{-5} mM choline (Fig. 6). There is a linear region

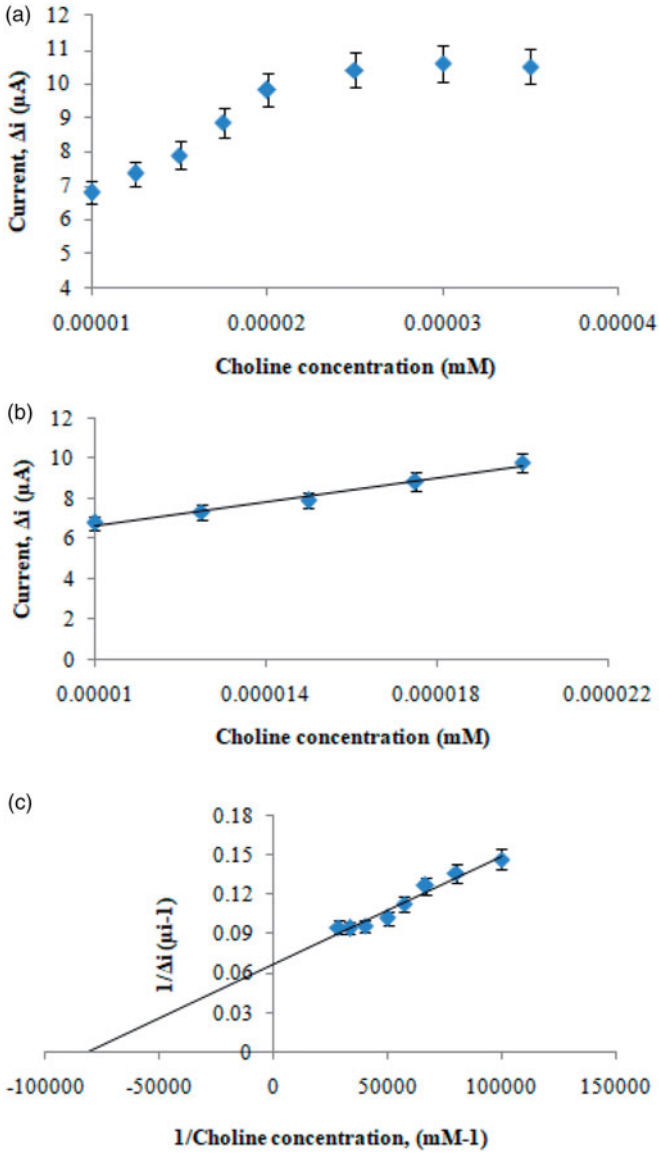


Figure 6. (a) The effect of choline concentration on the response of the biosensor (at 0.1 M pH 7.0 phosphate buffer, 25 C, −0.23 V operating potential). (b) The calibration curve of choline biosensor (at 0.1 M, pH 7.0 phosphate buffer, 25 °C). (c) Lineweaver–Burk plot of choline biosensor (at 0.1 M, pH 7.0 phosphate buffer, 25 °C).

between 1.0×10^{-5} and 2.0×10^{-5} mM ($R^2 = 0.9805$) (Fig. 6a). As shown in Fig. 6a, the calibration range of the biosensor has a very low concentration. This is advantageous for the determination of very small amounts of choline. Biosensors that allow low concentration determination are valuable. Analysis can be done by diluting a high concentration sample, but a biosensor with a high concentration range is not a remedy for analyzing substances at low concentrations.

Sensing limit of the biosensor was obtained as 1.0×10^{-6} mM (S/N:3). The limit of detection values of some choline biosensor studies in the literature is as follows; 1.0×10^{-4} mM,[7] 0.02 mM,[16] 8.0×10^{-3} mM,[29] 3.0×10^{-4} mM.[30] When these values are compared, it can be said that novel biosensor has a very low sensing limit value. Reply time of choline biosensor was 200 s. $K_{m(app)}$ value which is a special variable for enzymes was obtained from 1/[choline] to 1/Δi graph (Lineweaver–Burk graph) (Fig. 6b). $K_{m(app)}$ and I_{max} parameters were obtained as 1.19×10^{-5} mM and 14.92 μA, respectively. K_m shows the interest of an enzyme to its substrate. Lower K_m values are related to higher interest of enzyme to substrate. The $K_{m(app)}$ values reported in the literature were 0.89 mM and 228 μM.[21,26] It can be seen that PPy–PVS film utilized in this experiment increased interest of ChO to ACh. Because the $K_{m(app)}$ value, 1.19×10^{-5} mM, was lower than the $K_{m(app)}$ values in references.

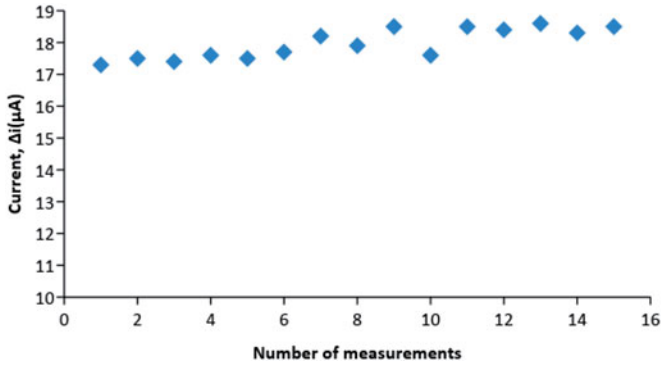


Figure 7. Operational stability of the biosensor in pH 7.0 phosphate buffer, at a −0.23 V operating potential, 25 C.

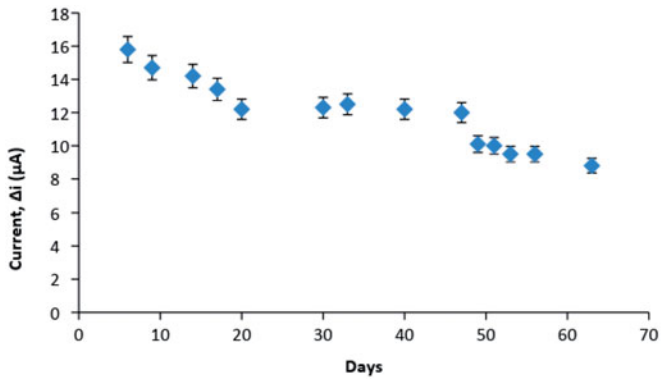


Figure 8. Storage stabilization of the biosensor in pH 7.0 phosphate buffer, at a −0.23 V operating potential, 25 C.

Operational and storage stability of choline biosensor

In biosensor studies, enzyme stability is very important. Generally, the enzyme is unstable in its aqueous solution while storage, so its activity progressively gets low.[31] Immobilized enzymes are much more stable than soluble enzymes and can be simply removed from the reaction medium. With the aim of testing repeatability of the biosensor, current alterations achieved after ensuing usage were drawn in a graph against number of experiments. Relative standard deflection achieved after 15 experiments at a steady choline concentration was found to be 2.64% (Fig. 7).

Depot endurance of the biosensor was detected for 63 days at steady choline concentration (5.0×10^{-5} mM). On the 63rd day, the activity loss of % 44.3 was determined. Both depot and application endurance results of the biosensor are very hopeful to constitute choline biosensor (Fig. 8).

Table 1. Summary of the biosensors made for the determination of choline in literature.

Carrier	Detection principle	Linear range (μM)	Immobilization technique	Optimum		K_m (μM)	Ref.
				pH	Temperature(°C)		
MWCNT film	H_2O_2 , +0.16 V	5–100	Entrapment	7.4	35	–	[32]
EACC multilayer film	Prussian Blue, 0.0 V	0.5–100	Adsorption	7.4	30	0.83 ± 0.1	[22]
HRP/CPE	Phenotiazine, 0.0 mV	0.5–70	Crosslinking	7.4	37	–	[7]
Ni-PB/BG nanocomposite film	Prussian blue, −0.05 V	0.45–100	Crosslinking	7.4	–	97	[5]
PPy/PVS film	H_2O_2, +0.3 V	0.1–1 and 0.01–1	Entrapment	9.0	60	0.558	[33]
PPy/pTS film	H_2O_2, +0.3 V	1–100	Crosslinking	9.5	65	135.5	[34]
PPy/PVS film	Toluidine blue O, −0.23 V	0.01–0.02	Crosslinking	7.0	30	0.019	This work

The effect of interferences

The influences of interferences on choline amperometric signals were investigated such as ascorbic acid (1.0×10^{-6} M), uric acid (3.0×10^{-6} M), dopamine (1.0×10^{-7} M), paracetamol (1.0×10^{-6} M), cysteine (1.0×10^{-6} M), and glucose (5.0×10^{-5} M) at 1.5×10^{-5} mM choline concentration. These agents were placed in measurement receptacle and diluted 200 times. All initiative types' effect becomes insignificant and no signal was observed with dilution influence. Therefore, it was decided that these substances did not attempt any initiative on choline analysis.

The choline detection in artificial blood specimen

An artificial blood specimen was prepared. This artificial blood specimen comprises concentrations of interference agents in human blood (ascorbic acid (1.0×10^{-6} M), uric acid (3.0×10^{-6} M), dopamine (1.0×10^{-7} M), paracetamol (1.0×10^{-6} M), cysteine (1.0×10^{-6} M), and glucose (5.0×10^{-5} M)). Choline concentration was constant at 1.5×10^{-5} mM. The artificial blood specimen was placed in measurement receptacle and diluted 200 times. Choline amount was determined after three experiments $(1.92 \pm 0.04) \times 10^{-5}$ mM using the calibration curve.

Conclusion

A novel ChO-based amperometric biosensor for the detection of choline was suggested in this article. TBO was chosen as electron transfer mediator. The calibration range of the biosensor has a very low concentration. This is advantageous for the determination of very small amounts of choline. Prepared biosensor has a very low sensing limit and an agreeable reply time. There were not any interference effects of different interferants. It was highly sensitive. Thus, choline determination can be made many times with this biosensor. It is considered suitable for multiple consecutive analyzes. It is simple to make ready and also very economical. Prepared biosensors for the detection of choline in reference are summarized and compared in this study (Table 1).

References

[1] He, S.; Wu, G. W.; Deng, H. H.; Liu, A.; Lin, X. H.; Xia, X. H.; Chen, W. Choline and Acetylcholine Detection Based on Peroxidase-like Activity and Protein Antifouling Property of Platinum Nanoparticles in Bovine Serum Albumin Scaffold. *Biosens. Bioelectron.* **2014**, *62*, 331–336.

[2] Jin, J.; Muroga, M.; Takahashi, F.; Nakamura, T. Enzymatic Flow Injection Method for Rapid Determination of Choline in Urine with Electrochemiluminescence Detection. *Bioelectrochem.* **2010**, *79*, 147–151.

[3] Thiagarajan, V.; Madhurantakam, S.; Sethuraman, S.; Rayappan, J. B. B.; Krishnan, U. M. Nano Interfaced Biosensor for Detection of Choline in Triple Negative Breast Cancer Cells. *J. Colloid Interface Sci.* **2016**, *462*, 334–340.

[4] Ulus, İ. H.; Cansev, M. Roles of Choline on Central and Peripheral Cholinergic Neurons and Cholinergic Neurotransmission. *Acibadem Univ. Health Sci. J.* **2010**, *1*, 68–80.

[5] Keihan, A. H.; Sajjadi, S.; Sheibani, N.; Moosavi-Movahedi, A. A. A Highly Sensitive Choline Biosensor Based on Bamboo-like Multiwall Carbon Nanotubes/Ionic Liquid/Prussian Blue Nanocomposite. *Sens. Actuators B Chem.* **2014**, *20*, 694–703.

[6] Phillips, M. M. Choline: Properties and Determination, Encyclopedia of Food and Healt. *Ref. Mod Food Sci.* **2016**, 73–78.

[7] Razola, S. S.; Pochet, S.; Grosfils, K.; Kauffmann, J. M. Amperometric Determination of Choline Released from Rat Submandibular Gland Acinar Cells Using a Choline Oxidase Biosensor. *Biosens. Bioelectron.* **2003**, *18*, 85–191.

[8] Chen, L.; Oates, K.; De Borba, B.; Rohrer, J. *Determination of Choline in Infant Formula and Other Food Samples by Ion Chromatography*; Thermo Fisher Scientific: Sunnyvale, CA, **2012**.

[9] Pomfret, E. A.; Costa, K. A.; Schurman, L. L.; Zeisel, S. H. Measurement of Choline and Choline Metabolite Concentrations Using High-Pressure Liquid Chromatography and Gas Chromatography-Mass Spectrometry. *Anal. Biochem.* **1989**, *18*, 85–90.

[10] Glick, D. Concerning the Reineckate Method for the Determination of Choline. *J. Biol. Chem.* **1944**, *156*, 643–651.

[11] Holm, P. I.; Ueland, P. M.; Kvalheim, G.; Ernst, A. L. Determination of Choline, Betaine, and Dimethylglycine in Plasma by a High-Throughput Method Based on Normal-Phase Chromatography–Tandem Mass Spectrometry. *Clin. Chem.* **2003**, *49*, 286–294.

[12] Langer, J. J.; Filipiak, M.; Ke¸Cińska, J.; Jasnowska, J.; Włodarczak, J.; Buładowski, B. Polyaniline Biosensor for Choline Determination. *Surf. Sci.* **2004**, *573*, 140–145.

[13] Marty, J. L.; Sode, K.; Karube, I. Amperometric Determination of Choline and Acetylcholine with Enzymes Immobilized in a Photocross-Linkable Polymer. *Anal. Chim. Acta* **1990**, *228*, 49–53.

[14] Lima, J. L. F. C.; Delerue-Matos, C.; Vaz, M. C. V. F. Enzymatic Determination of Choline in Milk Using a FIA System with Potentiometric Detection. *Analyst.* **2000**, *125*, 1281–1284.

[15] Haberfield, P.; Pessin, J. Proximate Charge Effects. 2.[1] Enthalpies of Solvent Transfer in the Choline-Anhydrocholine Equilibrium. *J. Am. Chem. Soc.* **1982**, *104*, 6191–6194.

[16] Pati, S.; Quinto, M.; Palmisano, F. Flow Injection Determination of Choline in Milk Hydrolysates by an

Immobilized Enzyme Reactor Coupled to a Selective Hydrogen Peroxide Amperometric Sensor. *Anal. Chim. Acta.* **2007**, *594*, 234–239.

[17] Qin, X.; Wang, H.; Wang, X.; Miao, Z.; Chen, L.; Zhao, W.; Shan, M.; Chen, Q. Amperometric Biosensors Based on Gold Nanoparticles-Decorated Multiwalled Carbon Nanotubes-Poly(Diallyldimethylammonium Chloride) Biocomposite for the Determination of Choline. *Sens. Actuators B Chem.* **2010**, *147*, 593–598.

[18] Pundir, S.; Chauhan, N.; Narang, J.; Pundir, C. S. Amperometric Choline Biosensor Based on Multiwalled Carbon Nanotubes/Zirconium Oxide Nanoparticles Electrodeposited on Glassy Carbon Electrode. *Anal. Biochem.* **2012**, *427*, 26–32.

[19] Chen, R. L. C.; Hsieh, B. C.; Matsumoto, K.; Cheng, T. J.; Yuu, G. Choline Biosensor Constructed with Chitinous Membrane from Soldier Crab and Its Application in Measuring Cholinesterase Inhibitory Activities. *J. Pharmaceut. Biomed.* **2007**, *45*, 673–678.

[20] Shi, H.; Song, Z.; Huang, J.; Yang, Y.; Zhao, Z.; Anzai, J.-I.; Osa, T.; Chen, Q. Effects of the Type of Polycation in the Coating Films Prepared by a Layer-by-Layer Deposition Technique on the Properties of Amperometric Choline Sensors. *Sens. Actuators B Chem.* **2005**, *109*, 341–347.

[21] Yang, M.; Yang, Y.; Yang, Y.; Shen, G.; Yu, R. Bienzymatic Amperometric Biosensor for Choline Based on Mediator Thionine in Situ Electropolymerized within a Carbon Paste Electrode. *Anal. Biochem.* **2004**, *334*, 127–134.

[22] Shi, H.; Yang, Y.; Huang, J.; Zhao, Z.; Xu, X.; Anzai, J. I.; Osa, T.; Chen, Q. Amperometric Choline Biosensors Prepared by Layer-by-Layer Deposition of Choline Oxidase on the Prussian Blue-Modified Platinum Electrode. *Talanta* **2006**, *70*, 852–858.

[23] Gros, P.; Durliat, H.; Comtat, M. Use of Polypyrrole Film Containing $Fe(CN)_6^{3-}$ as Pseudo-Reference Electrode: Application for Amperometric Biosensors. *Electrochim. Acta* **2001**, *46*, 643–650.

[24] Chaubey, A.; Gerard, M.; Singhal, R.; Singh, V. S.; Malhotra, B. D. Immobilization of Lactate Dehydrogenase on Electrochemically Prepared Polypyrrole–Polyvinylsulphonate Composite Films for Application to Lactate Biosensors. *Electrochim. Acta.* **2001**, *46*, 723–729.

[25] Aynacı, E.; Yaşar, A.; Arslan, F. An Amperometric Biosensor for Acetylcholine Determination Prepared from Acetylcholinesterase-Choline Oxidase Immobilized in Polypyrrole-Polyvinylsulpfonate Film. *Sens. Actuators B Chem.* **2014**, *202*, 1028–1036.

[26] Shimomura, T.; Itoh, T.; Sumiya, T.; Mizukami, F.; Ono, M. Amperometric Determination of Choline with Enzyme Immobilized in a Hybrid Mesoporous Membrane. *Talanta.* **2009**, *78*, 217–220.

[27] Rahman, M. A.; Park, D. S.; Shim, Y. B. A Performance Comparison of Choline Biosensors: anodic or Cathodic Detections of H_2O_2 Generated by Enzyme Immobilized on a Conducting Polymer. *Biosens. Bioelectron.* **2004**, *19*, 1565–1571.

[28] Shi, H.; Song, Z.; Huang, J.; Yang, Y.; Zhao, Z.; Anzai, J.; Osa, T.; Chen, Q. Effects of the Type of Polycation on the Amperometric Response of Choline Biosensors Prepared by a Layer-by-Layer Deposition Technique. *Mat. Sci. and Eng. C.* **2005**, *25*, 433–435.

[29] Hendji, A. N.; Bataillard, P. Jaffrezic-Renaul, N. Covalent Immobilization of Glucose Oxidase on Silanized Platinum Microelectrode for the Monitoring of Glucose. *Sens. Actuators B Chem.* **1993**, *15*, 127–134.

[30] Song, Z.; Huang, J. D.; Wu, B. Y.; Shi, H. B.; Anzai, J. I.; Chen, Q. Amperometric Aqueous Sol–Gel Biosensor for Low-Potential Stable Choline Detection at Multi-Wall Carbon Nanotube Modified Platinum Electrode. *Sens Actuators B Chem.* **2006**, *115*, 626–633.

[31] Özdemir, M.; Arslan, F.; Arslan, H. An Amperometric Biosensor for Choline Determination Prepared from Choline Oxidase Immobilized in Polypyrrole-Polyvinylsulfonate Film. *Artif. Cell Blood. Sub.* **2012**, *40*, 280–284.

[32] Şengül, B. Preparation of a New Biosensor with Immobilization of Uricase upon Polypyrrole-Paratoluenesulfonate. Master's Thesis, Gazi University Institute of Science, Ankara 59, **2015**.

[33] LóPez, M. S. P.; PéRez, P. H.; Cabarcos, E. L.; Ruiz, B. L. Amperometric Biosensors Based on Choline Oxidase Entrapped in Polyacrylamide Microgels. *Electroanalysis.* **2007**, *19*, 370–378.

[34] Qu, F.; Yang, M.; Jiang, J.; Shen, G.; Yu, R. Amperometric Biosensor for Choline Based on Layer-by-Layer Assembled Functionalized Carbon Nanotube and Polyaniline Multilayer Film. *Anal. Biochem.* **2005**, *344*, 108–114.