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Choline biosensor constructed with chitinous membrane from soldier crab and its application in measuring cholinesterase inhibitory activities

Bo-Chuan Hsieh [a], Kiyoshi Matsumoto [b], Tzong-Jih Cheng [a], Gyokurin Yuu [a], Richie L.C. Chen [a,*]

[a] *Department of Bio-Industrial Mechatronics Engineering, National Taiwan University, 136 Chou-Shan Road, Taipei City 106, Taiwan, ROC*
[b] *Institute of Food Biotechnology, Department of Bioscience and Biotechnology, Division of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka 812-8581, Japan*

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Abstract

An amperometric flow-injection choline biosensor was assembled utilizing natural chitinous membrane as the supporting material for biocatalyst immobilization, and the membrane was purified from Taiwanese soldier crab, *Mictyris brevidactylus*. The chitinous membrane (<50.0 μm in thickness) was covalently immobilized with choline oxidase (EC 3.1.1.17 from *Alcaligenes* sp.) and then attached onto the platinum electrode of an amperometric flow cell. The flow cell served as the choline sensing device of the proposed FIA system. The sensor signal (peak height of the FIAgram) was linearly related to choline concentration ($r = 0.999$ for choline up to 5.0 mM) with low detection limit (S/N > 3 for 10.0 μM choline) and high reproducibility (CV < 3% for 1.0 mM choline, $n = 7$). The system was proved to be useful in measuring cholinesterase inhibitory activities of synthetic chemicals or natural products.

Keywords: Alzheimer; Biosensor; Chitinous membrane; Cholinesterase; Pesticide; Soldier crab

1. Introduction

Inhibitory activity against acetylcholinesterase (AChE) is a general mechanism in developing cholinergic drugs and pesticides. Reversible inhibitors have been used in treatment of myasthenia gravis (e.g. neostigmine) and also Alzheimer's disease (e.g. Tacrine) [1–4]; the more toxic irreversible inhibitors such as organophosphates or carbamates are the major group of agricultural insecticides [5]. Therefore, analytical methods for detecting or characterizing anti-AChE activity are demanded in biomedical industries and also for public hygiene.

However, both the enzymatic products of AChE, choline and acetate, are optically and electrochemically inert for conventional chemical analysis [6–9], biosensor approaches were therefore considered [10]. Lacking proper analytical enzyme, acetate sensors [11,12] required multiple enzyme reactions, which complicated the sensor designs and the practical applications. Most biosensors for AChE inhibitory test were therefore based on immobilizing a stable and specific analytical enzyme from *Alcaligenes* sp., choline oxidase (ChOx; EC 1.1.3.17). With a durable and electrochemically inactive supporting material for enzyme immobilization, one can easily assemble an electrochemical anti-AChE sensor based on ChOx.

Chitinous materials (chitin and chitosan) are versatile in biomedical areas; the additional amino groups on their surface render the biomaterials a promising support for enzyme immobilization [13]. Most chitin and chitosan-based biosensors used reconstructed membrane cast with purified materials [14–19], but the mechanical characters are not satisfactory for long-term usage. In our recent studies, natural chitinous membranes from Taiwanese soldier crabs (*Mictyris brevidactylus*, Stimpson, 1858) were used for biosensor construction [20]. The natural membranes are chemically stable and mechanically tough, the amino group densities on the membrane surface are also sufficient for effective ligand-binding. The membrane itself is a physical barrier to prevent unwanted molecular attachments on the sensing electrode; the positively charged amino groups can

* Corresponding author. Tel.: +886 2 33665330; fax: +886 2 23627620.
E-mail address: rlcchen@ntu.edu.tw (R.L.C. Chen).

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either repel cationic interfering chemicals or pre-concentrate anionic analytes [21].

In the present approach, ChOx (EC 1.1.3.17) was immobilized on the natural chitinous membrane, and the resulting bioactive membrane was used to assemble an amperometric flow-injection choline biosensor. The choline sensing system was proven to be a fast and reliable tool for assessing the AChE inhibitory activities.

2. Experimental

2.1. Chemicals, enzymes and food extracts

Hydrochloric acid, sodium hydroxide, acetone and phosphoric acid (85%, w/v) were purchased from Union Chemical, Taiwan. Glutaraldehyde solution (25%, w/v), hydrogen peroxide (30%, w/v) and ascorbate were from Nacalai Tesque, Japan. Choline chloride, acetylcholine chloride and neostigmine bromide were purchased from Wako Pure Chemical. Other chemicals were of analytical grade and used as received.

Choline oxidase (EC 1.1.3.17 from *Alcaligenes* sp., 13 U/mg solid) and acetylcholinesterase (EC 3.1.1.7. from *Bovine erythrocyte*, 2.4 U/mg solid) were from Wako Pure Chemical, Japan. The enzymes were stored at −20 °C.

Dried and chopped samples of Ginkgo (*Ginkgo biloba*) leaves, grape (*Vitis coignetiae*) leaves, tea (*Camellia sinensis*) leaves and nelli (*Phyllanthus embelica*) fruits were homogenized and extracted for 12 h with methanol under mild stirring condition. The resulting suspensions were centrifuged, and the supernatants were freeze-dried to obtain water soluble powders. The extract powders were diluted with 0.2 M phosphate buffer (pH 8.0) for analysis. Deionized water with conductivity less than 1.0 μS/cm was used for sample dilution and reagent preparation.

2.2. Preparation of chitinous membranes

Taiwanese soldier crabs with carapaces of approximately 15.0 mm in diameter were collected from the intertidal sandy flats at Hsin-Chu, Taiwan and stored at −20 °C until use. Chitinous membranes, the exoskeletons of the crabs, were purified directly by a sequence of acid/base treatments [20,21] without dissection. The membranes for biosensing purposes were cut from the carapace parts for suitable size (12 mm × 8.0 mm, Fig. 1). The chitinous materials were stored in deionized water of 4 °C until use.

The thickness of purified chitinous membrane was measured using a micron micrometer (No. 293-561 N, Mitutoyo, Japan) fitted with a ratchet stop. Before the measurements, the membrane was rinsed with deionized water to ensure sufficient swelling of the biopolymer. The tensile strength and elongation of the chitinous membrane were mainly evaluated with a mechanical testing system (Model 4400, Instron Co., USA) and its modularized method, ASTM-D882 [22]. All testing specimens were cut into standard strips (9.0 mm length, 7.0 mm width) for normalization and measured at ambient temperature.

Fig. 1. Purified chitinous membrane of a Taiwanese soldier crab. Also shown are the membrane of the carapace part and a coin of 20 mm in diameter.

2.3. Enzyme immobilization

Prior to the experiment, alkaline glutaraldehyde solution (5%, v/v) was prepared by mixing 1.0 mL of 25% aqueous glutaraldehyde with 4.0 mL of carbonate buffer (0.2 M, pH 10.0). Chitinous membranes from the crabs' carapaces were immersed in the alkaline glutaraldehyde solution for ca. 2 h to activate the surface amino groups. After being rinsed with deionized water, the glutaraldehyde-treated chitinous membrane was immersed into enzyme solution (0.1 mg choline oxidase in 1.0 mL of 0.2 M phosphate buffer, pH 7.0) and kept at 4 °C for 16 h. The chitinous enzyme membrane was then rinsed with the mentioned phosphate buffer and stored at 4 °C until use.

2.4. Amperometric flow cell

The flow cell was composed of two resin blocks separated by a silicon septum (1.0 mm in thickness); these components were hold securely together by bolts and nuts (Fig. 2). On the surface of Resin block B inlayed the platinum working electrode (14.0 mm^2). Resin block A contains pathways (inlet and outlet, dotted line) for the carrier stream and a socket for Ag/AgCl reference electrode (RE-3V, BAS). The stainless outlet-pipe (1.0 mm i.d. tube for HPLC) served as the counter electrode for three-

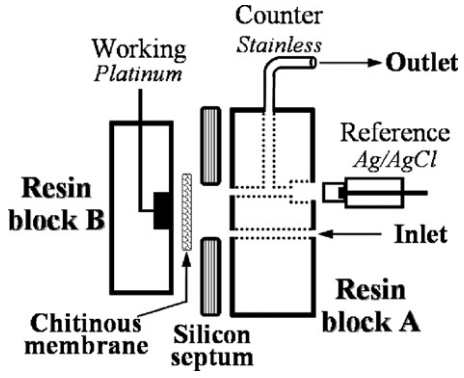

Fig. 2. Schematic diagram of the home-made amperometric flow cell. The assembling procedures are detailed in the experimental section.

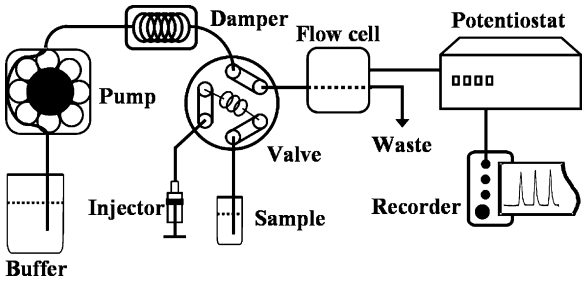

Fig. 3. Schematic diagram of the flow injection system.

electrode amperometric measurement. Carrier solution flowed through the oval opening (80 mm width and 150 mm length, as the electrochemical reaction chamber) of the silicon septum.

2.5. Flow-injection analytical system

The assembled electrochemical biosensing flow cell was incorporated into a flow-injection manifold (Fig. 3). With a controllable peristaltic pump (micro tube pump-mp3, EYELA), carrier solution (0.2 M phosphate buffer, pH 8.0) was continuously driven (typically, 1.5 mL/min) through a low pressure injection valve (model 5020, RHEODYNE) and then the flow cell. Sample solution was introduced into the carrier stream via the sample loop (ca. 20.0 μL) of the injection valve. Amperometric signal (0.6 V versus Ag/AgCl) was continuously monitored with a home-made potentiostat and a chart-pen recorder (R-50 series, RIKADENKI).

2.6. AChE inhibition ratio

All enzymes or chemicals were dissolved or diluted with 0.2 M, pH 8.0 phosphate buffer. As summarized in Table 1, different volumes of AChE (1.0 U/mL), the inhibitors, phosphate buffer and acetylcholine solutions (15.0 mM) were added into four different test tubes. After the two-stage 37 °C incubation to complete the reactions, the sensor responses of the four experimental groups (inhibition group, control group, blank 1 and blank 2) were used to calculate the inhibition ratio I%, Eq. (1):

$$I = \frac{(i_{ctrl} - i_{b1}) - (i_{inhi} - i_{b2})}{i_{ctrl} - i_{b1}} \times 100\% \quad (1)$$

where i_{ctrl} is the peak current of control group, i_{b1} the peak current of blank 1 group, i_{inhi} the peak current of inhibition group and i_{b2} is the peak current of blank 2 group.

Table 1
Reagent adding sequence and the procedure for AChE inhibitory assay

Reagent volume	Inhibition group	Control group	Blank 1 group	Blank 2 group
AChE	0.1 mL	0.1 mL	0 mL	0 mL
Inhibitor	0.1 mL	0 mL	0 mL	0.1 mL
Buffer	1.8 mL	1.9 mL	2.0 mL	1.9 mL
Incubation at 37 °C for 30 min				
Acetylcholine	1.0 mL	1.0 mL	1.0 mL	1.0 mL
Incubation at 37 °C for 90 min				

3. Results

3.1. Membrane preparation and mechanical properties

Clear chitinous membranes of at least 20 crabs were simultaneously obtained after the acid and alkaline treatment for approximately 3 days. Details about the results of membrane purification process were similar to the previous investigation [20]. Typically, about 30 mg of chitinous skin (membrane) was purified from a crab with carapace of ca. 15.0 mm in diameter; the deacetylation degrees were calculated spectrophotometrically to be around 25% [23]. The membranes were stable in alkaline, acidic and even organic medium (e.g. acetone used in removing the red pigments on the walking legs).

Membranes cut from the dorsal carapaces were measured to be less than 50 μm in thickness (20–50 μm for 20 crabs). The tensile strength of chitinous membrane (54.6 ± 3.3 kg/mm^2, $n = 5$) was higher than Parafilm® (43.1 ± 1.7 kg/mm^2, $n = 5$). The natural membrane is thinner than the laboratory-use thin film (Parafilm®, 120 μm in thickness), but with an even stronger mechanical strength. However, the breaking elongation of chitinous membrane (127.3 ± 4.6%, $n = 5$) was somewhat less than Parafilm® (378.1 ± 32.3%, $n = 5$). With the excellent mechanical properties, the thin elastic biomembranes can be easily handled and fit onto the sensing surface of a biosensor.

The electrochemical properties of this hydrophilic membrane were characterized in a previous report [21].

3.2. Selectivity improvement with electrode covered with the natural membrane

Ascorbate was thought to be the most problematic interfering chemical in electrochemical bioanalysis. The amperometric flow-injection signals (0.6 V versus Ag/AgCl) of ascorbate were measured with a bare platinum electrode and also with the same electrode covered with the natural chitinous membrane. The results were compared with the responses of H_2O_2 as in Fig. 4. As judged from the slopes of these calibration curves, the diffusion of H_2O_2 was somewhat tempered by the natural membrane, which led to a 60% decrease in sensitivity. The diffusion restriction effect of the positively charged chitinous membrane is more obvious for the negatively charged molecule, ascorbate.

3.3. Effect of ionic strength on selectivity

With increasing ionic strength of carrier solution, the ionic atmosphere of negatively charged ascorbate molecules will expand due to electrostatic shielding effect of counterions from background solution. Therefore, the transportation of ascorbate molecules through the positively charged chitinous membrane is expected to be slower in environment with higher ionic strength. Compared with the responses of H_2O_2, the amperometric signals of ascorbate obtained with the electrode covered with chitinous membrane significantly decreased as buffer concentration exceeded 0.2 M (Fig. 5). This leaded to a significant decrease in signal ratios at higher ionic strength. Considering both the sen-

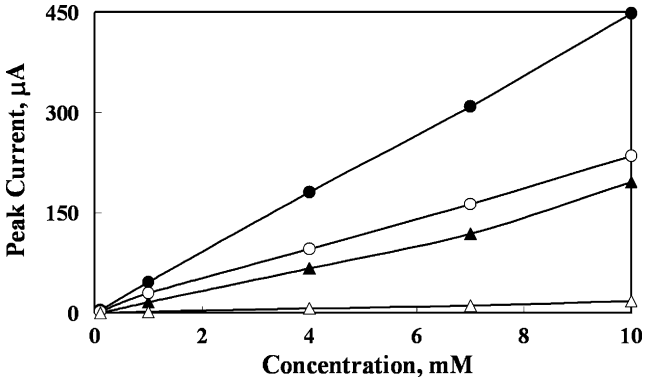

Fig. 4. Comparison of the amperometric flow-injection signals of H_2O_2 and ascorbate with bare or membrane-covered platinum electrode. Carrier solution: 0.2 M phosphate buffer, pH 8.0; applied voltage: 0.6 V vs. Ag/AgCl; flow rate: 1.5 mL/min. (●) Signals of H_2O_2 with bare electrode; (◯) signals of ascorbate with bare electrode; (▲) signals of H_2O_2 with electrode covered with chitinous membrane; (△) signals of ascorbate with electrode covered with chitinous membrane. Each datum is the average of seven repetitive experiments with standard deviation smaller than the symbol.

sor selectivity and enzyme stability, 0.2 M carrier solution was selected in this study.

3.4. Dose-dependent biosensor responses

Along with the carrier flow, injected segments (20.0 μL) of sample solution were delivered into the electrochemical flow cell with the chitinous membrane immobilized with choline oxidase (ChOx). Choline was oxidized rapidly by dissolved oxygen on the outer surface of the chitinous membrane. The evolved hydrogen peroxide diffused through the chitinous membrane and finally reached the positively polarized (0.6 V versus Ag/AgCl) platinum electrode:

$$\text{choline} + 2O_2 \xrightarrow{\text{ChOx}} \text{betaine} + 2H_2O_2 \quad (2)$$

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \text{ (platinum electrode)} \quad (3)$$

The carrier solution (0.2 M phosphate buffer, pH 8.0) served as both the pH buffer and supporting electrolytes for the electrochemical bioanalytical system. Oxidative currents of H_2O_2 were monitored, and typical FIAgrams were recorded. The sample throughput was higher than 20 determinations per h when carrier flow rate was set at 1.5 mL/min. The calibration curve for choline passes through the origin with good linearity ($R^2 = 0.9993$), which allows one-point calibration for rapid analysis. The linearity was up to 5.0 mM, and the S/N ratio of the signals from 10.0 μM choline was higher than 3. The relative standard deviations ($n = 7$) for determination of 1.0 mM choline was less than 3%. The electrochemical choline sensitivity of the FIA system was calculated to be 14.2 nA/μM cm^2. The high sensitivity persisted for months, the decrease in sensitivity was less than 10% after two months of daily usage (data not shown).

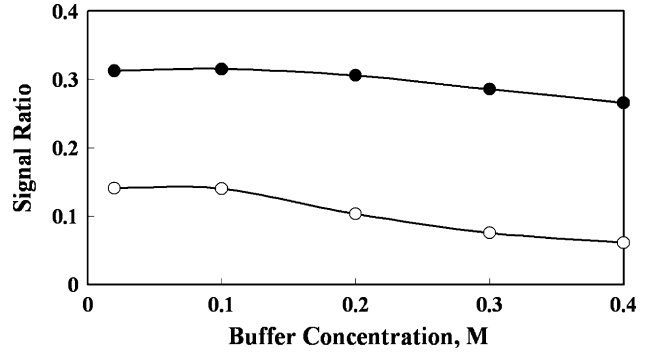

Fig. 5. Effects of buffer concentration on current reduction by chitinous membrane. The ordinate is the signal ratio of peak current obtained with membrane-covered electrode to that with bare electrode. (●) signal ratio of H_2O_2; (◯) signal ratio of ascorbate. Other conditions are the same as Fig. 4.

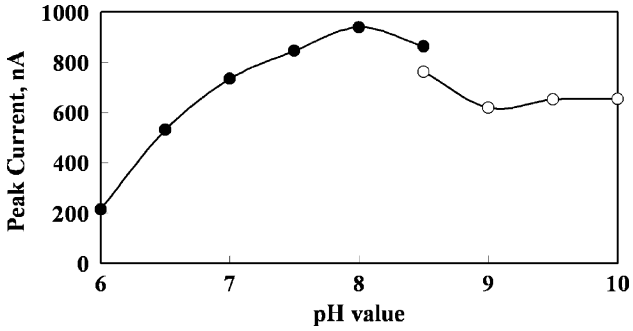

Fig. 6. Effect of pH on sensor sensitivity. The peak currents were measured by injecting 1.0 mM choline in different carrier solutions. (●) The 0.2 M phosphate buffer; (◯) 0.2 M carbonate buffer. Other conditions are the same as Fig. 4.

3.5. pH optimization

The pH dependency of the sensor response was studied with 0.2 M phosphate buffer and carbonate buffer (Fig. 6), the pH profile was similar to a recent report [24]. The pH optimum was found to be around pH 8.0, phosphate buffer.

3.6. Measuring AChE inhibitory activity

Acetylcholinesterase (AChE, 0.1 U) catalyzes the following reaction, and the reaction can be quantified by the choline released:

$$\text{acetylcholine} + H_2O \xrightarrow{\text{AChE}} \text{choline} + \text{acetic acid} \quad (4)$$

With the aforementioned biosensing system, the tabulated protocol (Table 1) and Eq. (1), the dose-dependent inhibition curve of a chemical can be obtained and its IC_{50} can be estimated. Typical sigmoid inhibition curves of neostigmine were obtained with initial acetylcholine concentration higher than 1.67 mM (Fig. 7), 5.0 mM acetylcholine was used thereafter for IC_{50} measurement. The estimated IC_{50} (500 nM from Fig. 7) is comparable with a documented result [25].

3.7. Effect of temperature on IC_{50}

Conformation and the kinetics of an enzyme are prone to be affected by temperature. As from Fig. 8, the IC_{50} are exten-

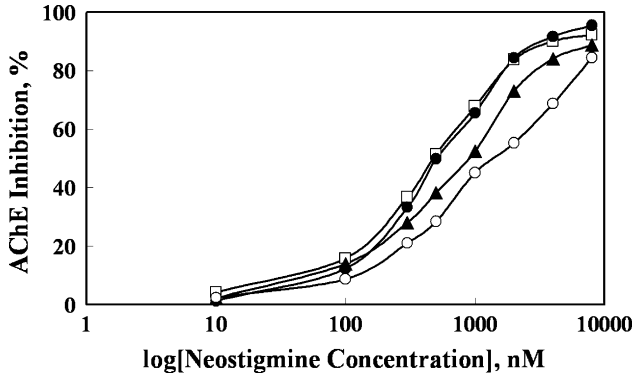

Fig. 7. AChE inhibition curves of neostigmine with different initial acetylcholine concentrations. (□) The 10.0 mM acetylcholine; (●) 5.0 mM acetylcholine; (▲) 1.67 mM acetylcholine; (○) 0.5 mM acetylcholine. The above concentrations are the final acetylcholine concentrations in the second incubation of Table 1. The inhibition ratios were calculated by Eq. (1). Each datum is the average of five repetitive experiments with standard deviation smaller than the symbol. Other conditions are the same as Fig. 4.

sively elevated by lowering the incubation temperature. The temperature-dependency partially revealed the homeotherm origin of the enzyme (from bovine erythrocyte).

3.8. Screening anti-AChE components from natural materials

The methanolic extracts of four plants were found to possess AChE inhibitory activities (Table 2). The extracts were diluted with phosphate buffer (0.2 M, pH 8.0) to different extents to obtain the inhibition ratios. The extracts of tea leaves and nelli fruits may contain residual esterase with acetylcholine hydrolyzing activity, the differences in i_{b1} and i_{b2} are therefore not negligible as compared with that of ginkgo leaves extract and grape leaves extract. Judging from the approximate IC_{50}, ginkgo leaves seems to be the most pharmacologically potent food stuff for developing medicine or nutraceuticals. Further purification procedures should be undertaken to identify the active ingredients in these materials.

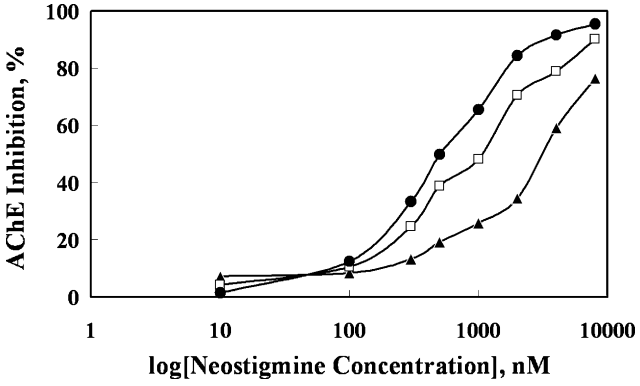

Fig. 8. Effect of temperature on AChE inhibition curve. (●) 37 °C; (□) 27 °C; (▲) 17 °C. Initial acetylcholine concentration was 5.0 mM. Other parameters are the same as Fig. 7.

Table 2
AChE inhibition by the extracts of several plants

Sample extracts	Concentration (mg/mL)	AChE inhibition (%)
Ginkgo leaves	1.5	40.1
	1.7	49.3
	2.0	64.1
	3.0	78.3
Grape leaves	1.0	15.8
	2.0	31.1
	3.0	51.6
	4.0	69.5
Tea leaves	9.0	37.8
	10.0	50.9
	11.0	59.0
	12.0	67.8
Nelli fruits	10.0	29.4
	11.0	37.0
	12.0	43.7
	13.0	50.4

4. Discussion

Although most biosensors [26–28] for anti-AChE test were constructed by co-immobilizing AChE and ChOx on their sensing devices, the denaturation of AChE during immobilization process or after the reaction with inhibitors led to a lower sensitivity or even false results especially after repetitive tests. Using free AChE and with choline oxidase immobilized were thus suggested [29–32], this study provided also a practical protocol for calculating inhibition ratios.

Choline oxidase from *Alcaligenes* sp. is stable and specific for analytical purposes. Unlike the other commercialized enzymes such as those from *Arthrobacter globiformis*, the enzyme is free from product inhibition, other similar compounds such as glycine or betaine aldehyde will not alter the enzymatic reaction. The analytical enzyme was immobilized on the surface of an ideal supporting material, the chitinous membrane from soldier crab; the performances of our system are therefore expected.

The dynamic range and reproducibility of the proposed sensing system are generally higher than the documented choline sensors [33–37]; the detection limit is comparable with the most recent approaches using electrodes modified with Prussian blue [38] or even carbon nanotube [39,40]. The outstanding durability [20] further expands its practical use. Possible applications [41] such as screening cholinergic or anti-Alzheimer chemicals, detecting residual pesticides in agricultural products and also diagnosing abnormality in liver functions are under investigations.

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