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All-solid-state Potentiometric Biosensors Based on Electropolymerized Poly(3,4-ethylenedioxythiophene) as Solid Contact for Acetylcholine Determination in Artificial Cerebrospinal Fluid

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All-solid-state ion-selective electrodes are a feasible method to realize the determination of neurotransmitters. Herein, an all-solid-state potentiometric acetylcholine biosensor was investigated. Poly(3,4-ethylenedioxythiophene) doped with poly(sodium 4-styrenesulfonate) as solid contact was electropolymerized on a gold disk covered with an acetylcholine selective membrane containing heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin as ionophore. The biosensor could work stably within a pH range from 6.5 to 8.5. The optimized acetylcholine biosensor had a good linear correlation over an acetylcholine concentration range of 10^{-5} M to 10^{-2} M with a slope of 58.2 ± 1.2 mV/decade and the detection limit was 6.01 ± 1.02 μM. The interference resistance, repeatability and stability were evaluated. The biosensor was also applied to detect the dynamic change of acetylcholine in artificial cerebrospinal fluid. The results showed that the newly developed biosensor had high sensitivity, a rapid response and a long lifetime. The technology is promising for fabricating acetylcholine biosensors with various suitable structures to satisfy the requirements of neurophysiological researches.

Keywords: Acetylcholine biosensor; All-solid-state; Ion-selective electrode; Artificial cerebrospinal fluid

1. INTRODUCTION

Acetylcholine (ACh) is an important neurotransmitter in the human nervous system and it widely exists in cerebrospinal fluid, intercellular fluid and blood [1]. ACh is associated with learning, memory, attention and muscle contraction [2, 3]. Clinical evidence has indicated that some neuropsychiatric disorders, such as Alzheimer's disease, myasthenia gravis and Parkinson's disease, are coursed by

dysfunctional ACh regulation [4, 5]. The etiologies are still unclear. Therefore, more attentions need to be paid to detecting ACh in vivo and in vitro.

There are currently several methods for detecting ACh, such as high-performance liquid chromatography detection with microdialysis samples [6, 7], spectroscopic detection with different fluorescent markers [8-10] and electrochemical detection [11-16]. Among these methods, electrochemical biosensors offer several advantages, such as rapidity, real-time monitoring, operational simplicity, portability and low cost. Since acetylcholinesterase (AChE) can catalyze the hydrolysis process of ACh, most electrochemical biosensors use immobilized AChE as the sensitive material. This type of biosensor is detected by an amperometric device. However, due to the high operating potential used in the process, electroactive compounds such as dopamine and ascorbic acid will substantially affect the measurement [17]. In addition, biosensors with immobilized enzymes are difficult to store and have a short shelf life. In contrast, ion-selective electrodes (ISEs) can detect the concentration or activity of charged substances by directly measuring the membrane potential, and ACh is a cationic neurotransmitter in aqueous solutions [18]. Such sensors do not need additional potential and can be easily stored for a long time [19]. In addition, ISEs have no effect on the detection environment and are suitable for colored or turbid solutions.

Conventional ISEs with internal filling solutions that satisfy the needs of different applications are difficult to fabricate and do not have long shelf-lives. As time goes on, conventional ISEs will measure inaccurately because of the loss of internal filling solutions, which is unsuitable for long term detection. To replace the internal filling solutions, electroactive materials called conducting polymers, which can fulfill the ion-to-electron transduction in solid state, are used between the interfaces of electronic conductors and selective membranes. A number of materials can be used as conducting polymers, such as polypyrrole, polythiophene, polyaniline and their derivatives [20, 21]. Among electroactive materials, poly(3,4-ethylenedioxythiophene) (PEDOT) doped with poly(styrenesulfonate) (PSS) has been found to be a particularly stable conducting polymer due to its low sensitivity to O_2 and CO_2 (pH) [22, 23].

In the present work, an all-solid-state ACh biosensor is studied. PEDOT doped with PSS was electropolymerized on a gold disk to fabricate the solid contact. The solid contact layer was covered by an ACh sensitive membrane by the dipping method. The performances of the ACh biosensor were tested. The biosensor allows the stable real-time measurement of artificial cerebrospinal fluid. Based on the fabrication method, it is expected that the ACh biosensor can be fabricated into different suitable structures to be inserted into mammalian nervous tissues to monitor the concentration of ACh in different physiological and psychological states.

2. EXPERIMENTAL

2.1 Materials and apparatus

High molecular weight poly(vinyl chloride) (PVC), heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin, potassium tetrakis[3,5-bis(trifluoromethyl)phenyl]borate (KTPB), 2-nitrophenyl octyl ether (NPOE), 3,4-ethylenedioxythiophene (EDOT), poly(sodium 4-styrenesulfonate) (NaPSS), acetylcholine chloride

(ACh), choline chloride (Ch), dopamine hydrochloride (DA), ascorbic acid (AA), D-(+)-glucose (GC) and urea (Ur) were purchased from Sigma Fluka. Other chemicals, including tetrahydrofuran (THF), sodium hydroxide (NaOH), sodium chloride (NaCl), potassium dihydrogen phosphate (KH_2PO_4), potassium chloride (KCl) and calcium chloride ($CaCl_2$) were purchased from Sinopharm Chemical Reagent Co., Ltd, China. Artificial cerebrospinal fluid (ACSF) was obtained from Shanghai Canspec Scientific & Technology Co., Ltd, China. All chemicals were of analytical reagents grades and deionized water was used throughout.

To obtain phosphate buffer solutions (PBS) at different pH values, 100 ml of 0.01 M KH_2PO_4 and an appropriate volume of 0.01 M NaOH were mixed and diluted to 200 ml with deionized water [24]. AChCl (0.18166 g) was dissolved in 10.0 ml of PBS at pH 7.4 to prepare a 0.1 M ACh stock solution. ACh solutions of other concentrations were prepared by diluting the stock solution with PBS. All the prepared solutions were stored at 4 ℃.

Potentiometric measurements were recorded with a CHI660E Electrochemical Workstation (CH Instrument Inc., USA). Gold disk electrodes (2 mm in diameter), platinum (Pt) disk electrodes (2 mm in diameter) and Ag/AgCl reference electrodes (Tianjin Aidahengsheng Technology Co., Ltd), containing 3.0 M KCl solution were used. The working area of the gold disk electrode was 3.14 mm^2.

2.2 Fabrication of the acetylcholine biosensor

Before immobilizing the PEDOT/PSS layer, 0.3 μm Al_2O_3 slurries were applied to polish the surface of the gold disk, and the disk was sequentially washed with 50.0 vol% ethanol solution, 5.0 wt% sulfuric acid and deionized water by ultrasonication. Then, the electrode was dried with N_2 and stored in a drying oven.

The supporting electrolyte containing EDOT and NaPSS was prepared by stirring for 12 h and then deaerating with N_2 for 10 min. A conventional three electrode electrochemical cell was applied with the gold disk electrode as working electrode, a Pt disk electrode as the counter electrode and a Ag/AgCl electrode as the reference electrode. The PEDOT/PSS conducting polymer was electropolymerized by chronopotentiometry in an electrolyte. After that, the surface of the PEDOT/PSS layer was washed with deionized water and dried in a drying oven for 12 h.

The ACh selective membrane solution was prepared by dissolving an ionophore (heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin), the membrane matrix (PVC), a plasticizer (NPOE) and an anion-exchanger (KTPB) in THF as the solvent. The ratio of PVC to THF was 1:10 (w/vol). The ACh selective membrane solution was mixed to homogeneity and stored in a refrigerator at 4 ℃. Then 4 μl of the ACh selective membrane solution was dropped on the PEDOT/PSS layer. The prepared biosensor was stored in a drying oven for 24 h. The ACh biosensor is shown in Fig. 1.

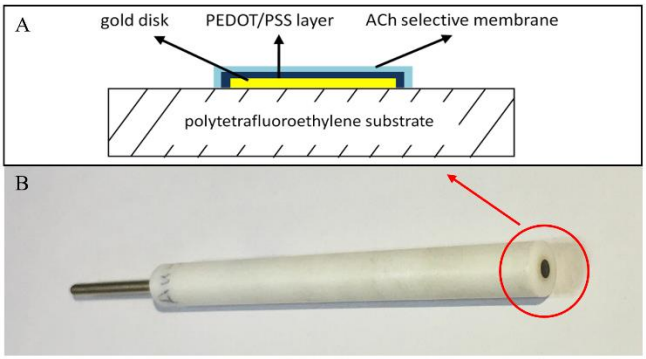

Figure 1. Structure (A) and picture (B) of ACh biosensor.

2.3 Evaluation of the potentiometric measurements

Before the measurements, the biosensor was immersed in deionized water until a steady potential was reached. The ACh biosensor and reference electrode worked as a two-electrode system for potentiometric measurement at approximately 25 ℃. The potentiometric data were related to the ACh concentrations in the test solutions. According to IUPAC recommendations, the selectivity coefficient was determined by the fixed interference ion method (FIM) [25]. The selectivity coefficient ($K_{A,B}^{pot}$) was calculated from the following equation:

$$K_{A,B}^{pot} = \frac{a_A}{a_B^{Z_A/Z_B}} \qquad (1)$$

Where a_A, a_B, Z_A and Z_B present the concentration of ACh and interference and the charge numbers of ACh and interference, respectively.

In the experiments, a variety of test solutions with different ACh concentrations were prepared and measured. The measurements were performed from low concentration to high concentration. The ACh calibrations were fitted to the Nernst equation. After the measurements, the biosensor was washed with deionized water and stored in a drying oven.

3. RESULTS AND DISCUSSION

3.1 Optimization of the acetylcholine biosensor

The supporting electrolyte was prepared by dissolving 1 mmol of EDOT and 10 mmol of NaPSS in 100 ml of deionized water [26, 27]. The PEDOT/PSS layer was electropolymerized by chronopotentiometry with a current density of 0.2 mA/cm^2. Six gold disk electrodes were prepared with different polymerization times (600 s, 1200 s, 1500 s, 1800 s, 2100 s and 2400 s). The formulation of the acetylcholine selective membrane remained consistent. Based on the slope and the detection limit of the ACh biosensor as the evaluation criteria, the optimal electropolymerization time was 1800 s. An SEM image of the PEDOT/PSS layer is shown in Fig. 2A. The PEDOT/PSS layer was dense and smooth

with no fractures or cavities, which is consistent with the results of previous studies [28, 29].

The ACh selective membrane was optimized by changing the content of the ionophore and keeping the mass ratio of the other three components the same [18, 30]. The slope, detection limit, linearity and selectivity coefficient of choline at 0.5 mM as the common interferent were used as the evaluation criteria for the performance of the selective membrane. Six ACh biosensors were prepared with six different formulations of the selective membrane and the performances of the biosensors are shown in Table 1. The experimental results indicated that the performances of the biosensors were improved by the addition of ionophore. When the mass ratio of ionophore was increased beyond 1.0 %, the performances were not significantly improved. Overall, the optimized ACh selective membrane consisted of 1.0 % (w/w) heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin, 32.9 % (w/w) PVC, 65.6 % (w/w) NPOE and 0.5 % (w/w) KTPB. According to the SEM image of the ACh selective membrane, the surface of the membrane presented regular and uniform folds, as shown in Fig. 2B.

A comparison of the ACh biosensor in this paper with other similar reported biosensors is presented in Table 2. According to the Nernst response characteristics, the Nernst theoretical slope of the ACh ISE is 59.2 mV/decade in the ideal state at 25 ℃ since ACh has a positive charge [18, 31]. As shown in the table, biosensor No.1 had the best slope and detection limit. However, due to the lack of ACh sensitive ionophore in the selective membrane [18], the selectivity of the biosensor No. 1 was worse than those of the other sensors [14, 32, 33], so it was not suitable for the detection of ACh in complex solutions. Compared with the ACh biosensor developed in this paper, biosensor No. 2 uses the same ionophore, but the formulations of the ACh selective membranes were different, resulting in different performances. The biosensor No. 2 was based on the conventional ISE structure and has a lifetime of 21 days [32], which is much shorter than that of the all-solid-state ACh biosensor developed in this paper. The performances of biosensors No. 3 and No. 4 are little worse than that of the biosensor in this paper. In conclusion, the comprehensive performances of the ACh biosensor designed in this paper is satisfactory.

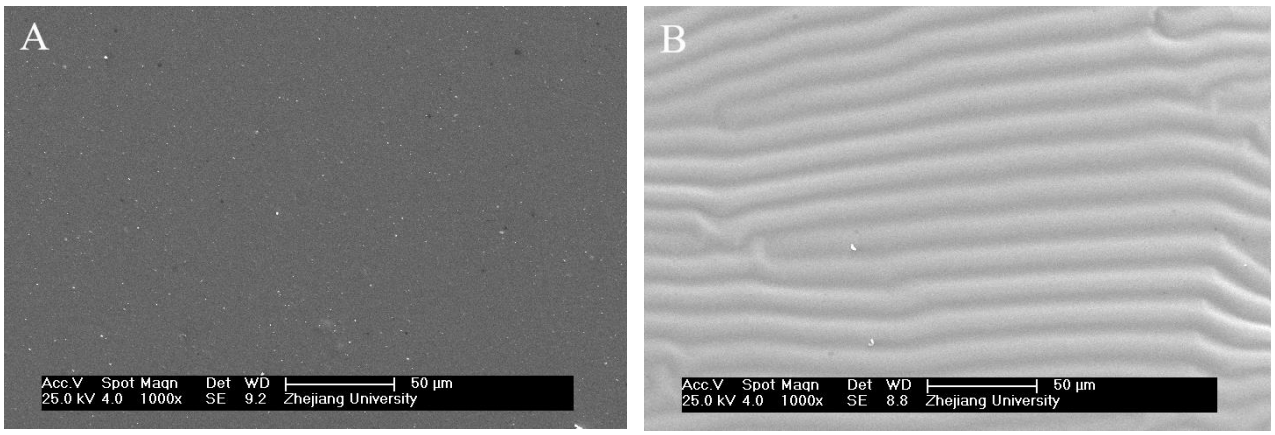

Figure 2. SEM image of PEDOT/PSS layer (A) and ACh selective membrane (B).

Table 1. Performances of the ACh biosensors with selective membrane of different formulations.

ACh biosensors	Formulations of selective membrane (w/w)				Slope (mV/decade)	Detection limit (μM)	Linearity	Selectivity coefficient of choline (log K)
	PVC	NPOE	KTPB	β-CD				
No. 1	33.2	66.3	0.5	0	57.1	8.23	0.9973	-1.03
No. 2	33.0	66.0	0.5	0.5	56.6	6.51	0.9985	-1.55
No. 3	32.9	65.8	0.5	0.8	56.8	6.13	0.9973	-1.68
No. 4	32.9	65.6	0.5	1.0	58.2	6.01	0.9980	-1.81
No. 5	32.8	65.5	0.5	1.2	58.1	6.07	0.9979	-1.81
No. 6	32.7	65.3	0.5	1.5	58.0	6.05	0.9986	-1.82

Table 2. Comparison with other reported biosensors for acetylcholine detection

ACh biosensor	Formulation of ACh selective membrane				Slope (mV/ decade)	Detection limit (μM)	Ref.
	ionophore	plasticizer	exchanger	matrix			
No. 1		NPOE	KTPB	PVC	59.1	0.15	[18]
No. 2	Ionophore I	NPOE	KTFPB	PVC	55.6	2.7	[32]
No. 3	Ionophore II	NPOE	TCP	PVC	57.4	10	[14]
No. 4	Ionophore III	NPOE	TKB	PVC	61.4	10	[33]
No. 5	Ionophore I	NPOE	KTPB	PVC	58.2	6.01	This work

Ionophore I: heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin; Ionophore II: 2,3,6-trimethyl o-β-cyclodextrin; Ionophore III: 2,6-didodecyl-β-cyclodextrin; KTFPB: potassium tetrakis(4-florophenyl) borate; TCP: tricresylphosphate; TKB: sodium tetrakis[3,5-kis(trifluromethy1)phenylboratel].

3.2 Calibration of the acetylcholine biosensor

To calibrate the ACh biosensor, eleven test solutions with different ACh concentrations, from 10^{-7} M to 10^{-1} M, were sequentially measured for three times. The response curve of the ACh biosensor is shown in Fig. 3, in which the abscissa represents the logarithm of the ACh concentration and the ordinate indicats the potential difference between the ACh biosensor and the reference electrode. The biosensor revealed a good linear response ($R^2 = 0.9997$) in a range of 10^{-5} M to 10^{-2} M, with a slope of 58.2 ± 1.2 mV/decade. By optimizing the selective membrane and PEDOT/PSS layer, the slope of the ACh biosensor in this paper was significantly improved from a slope of 54.0 mV/decade in our previously published work [11]. The linear curve was close to the Nernstian response. The detection limit was 6.01 ± 1.02 μM, which was calculated according to the IUPAC recommendations [25].

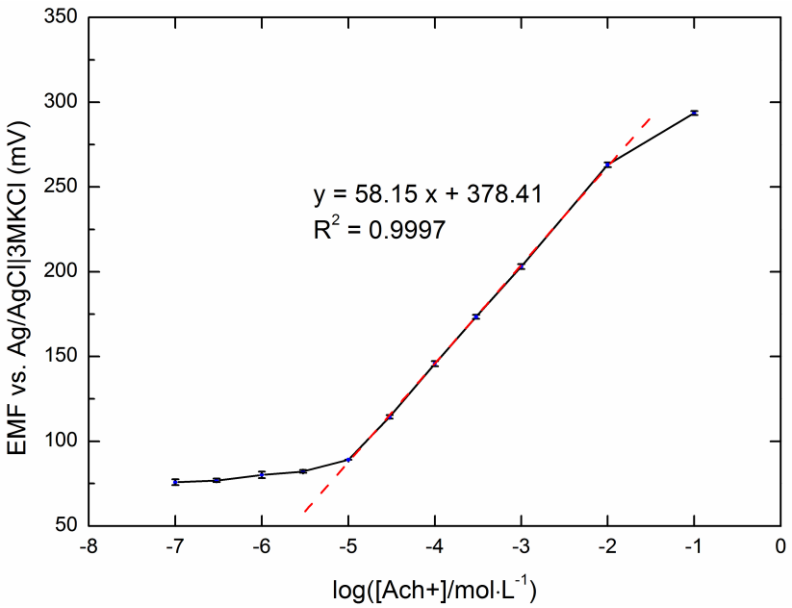

Figure 3. Calibration curve of the ACh biosensor based on standard ACh solutions.

3.3 Effect of pH

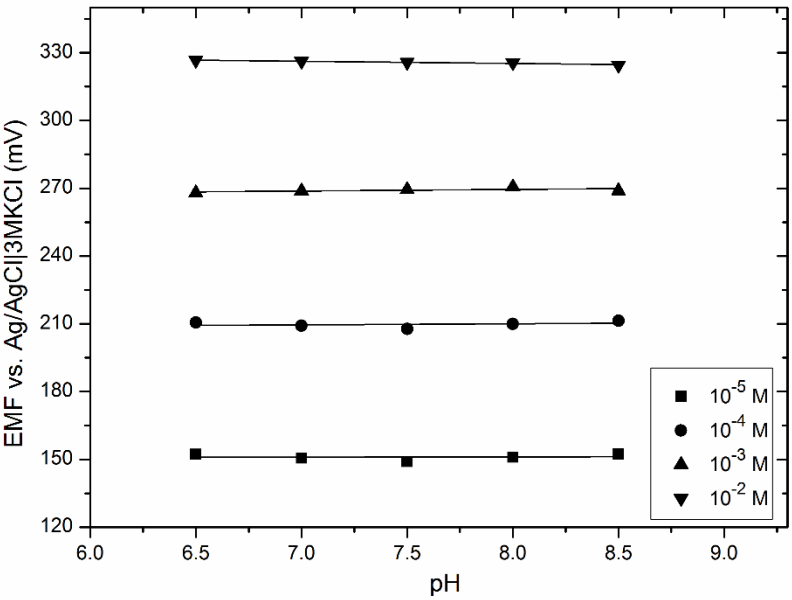

Figure 4. Effect of pH on the response to standard ACh solutions.

The effect of pH on the response of the ACh biosensor was examined. Considering that the pH range of human interstitial fluid is 6.6 - 7.6 [34], the pH test range was from 6.5 to 8.5. The tested ACh concentration range was from 10^{-5} M to 10^{-2} M. The results are shown in Fig. 4. The detection voltages of the ACh biosensor remained basically unchanged at the same concentrations. The ACh biosensor revealed a good linear response with an average slope of 58.2 mV/decade at different concentrations of the same pH from 6.5 to 8.5. The results showed that the ACh biosensor could work stably and not be affected by pH variations in the interstitial fluid.

3.4 Selectivity coefficients

The selectivity coefficients of ACh biosensor for interferences were calculated by the fixed interference ion method. K^+, Na^+, Ca^{2+}, Ch, DA, AA, GC and Ur were selected as interferences, as they are commonly found in cerebrospinal fluid. The results are shown in Table 3. It can be seen from the table that the ACh biosensor had good response characteristics in the presence of these interferences. The ionophore heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin, which was applied in this ACh selective membrane, has a toroidal cavity that exhibits molecular recognition with three types of interactions, a conventional hydrophobic effect, directed -N-H···O and N-C-H···O hydrogen bonding and van der Waals forces [11]. The selectivity of the ACh biosensor was better than those of other ACh sensors with different sensitive membrane formulations with other cyclodextrin derivatives as the ionophores or without ionophores [14, 18, 32, 33, 35]. Compared with other interferences, Ch revealed the greatest interference. This is mainly because Ch and ACh have similar chemical structures and properties. In the experiment, the Ch concentration was fixed to 0.5 mM. However, the Ch content in human cerebrospinal fluid was very low (0 to 2 μM). At this concentration, Ch had little effect on the measurement of ACh by the biosensor.

Table 3. Selectivity coefficients of ACh biosensor.

Interferents	Concentrations of the interferents	Logarithm of selectivity coefficients
K^+	0.1 M	-3.55
Na^+	0.1 M	-4.42
Ca^{2+}	0.1 M	-5.23
Ch	0.5 mM	-1.81
DA	0.01 M	-3.96
AA	0.01 M	-3.65
GC	0.1 M	-4.96
Ur	0.1 M	-5.01

3.5 Reproducibility and stability

Table 4. Reproducibility of the ACh biosensors.

Concentration of ACh (mM)	Detection results of ACh biosensors Nos. 1-3					
	No. 1		No. 2		No. 3	
	Mean (mM)	R.S.D. (%)	Mean (mM)	R.S.D. (%)	Mean (mM)	R.S.D. (%)
0.010	0.010	5.000	0.010	7.000	0.010	4.000
0.100	0.098	4.257	0.100	8.125	0.101	4.381
1.000	0.969	7.325	1.021	8.456	1.016	6.143

To test the reproducibility of the ACh biosensor, three biosensors were fabricated to measure

three standard solutions. Every sample was detected by each biosensor for three times, as shown in Table 4. The results detected by the same ACh biosensor at different concentrations were close to the calibration values. The data from three biosensors for the same ACh solution were similar and the relative standard deviation was within 3 %, which revealed that the reproducibility of the ACh biosensor prepared by electropolymerization is better than that of the sensor prepared in our previous work [11]. The results indicate that the reproducibility of the biosensors is acceptable.

The drift of ACh biosensor was also studied. A standard ACh solution of 10^{-5} M was measured over a period of 20 minutes. The maximum fluctuation in the potential in 20 minutes was 0.6 mV, which meant there was no need for drift correction during detection.

To test the long-term stability, the ACh biosensor was used to measure ACh solutions with six different concentrations every few days. After the measurements, the ACh biosensor was washed with deionized water and stored in drying oven. Using the acquired data, calibration curves were drawn and the slopes were calculated. The slopes of the calibration curves changed by approximately 5.0 mV/decade within 50 days, which indicates that the stability is satisfactory, as shown in Fig. 5.

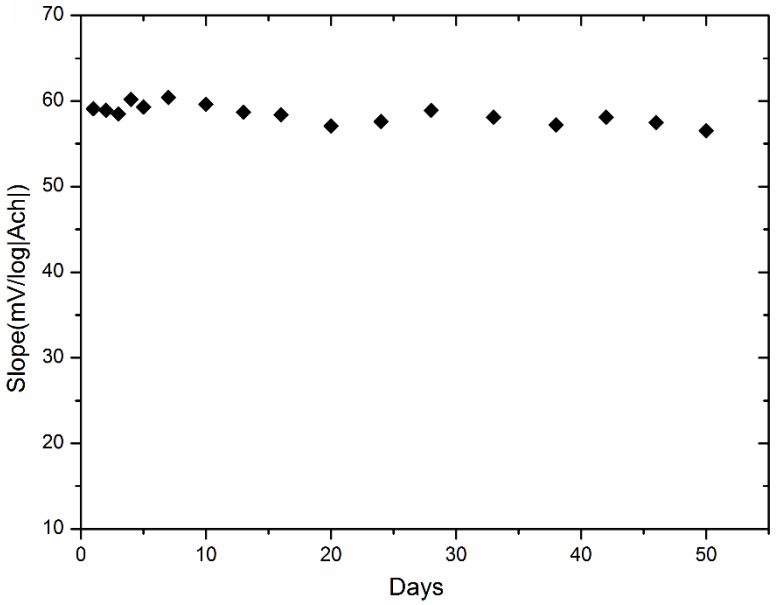

Figure 5. Long-term stability of ACh biosensor.

3.6 ACh detection in artificial cerebrospinal fluid

As a neurotransmitter, ACh is rapidly transmitted in brain tissue fluid. The compositions of the tissue fluids are similar to those of cerebrospinal fluid, including K^+, Na^+, Mg^{2+}, Ca^{2+}, Cl^-, $H_2PO_4^-$, HCO_3^-, GC, AA and Ur [36]. The dynamic response of the ACh biosensor was measured in artificial cerebrospinal fluid (ACSF), as shown in Fig. 6. During the experiment, a certain volume of high concentration ACh solution was added into the ACSF every 1 min with continuous magnetic stirring at 400 rpm. It can be seen from the figure that the ACh biosensor responded rapidly to the addition of ACh and stabilized within 3 s, which indicated that the biosensor was suitable for detecting rapid changes in the ACh concentration in ACSF.

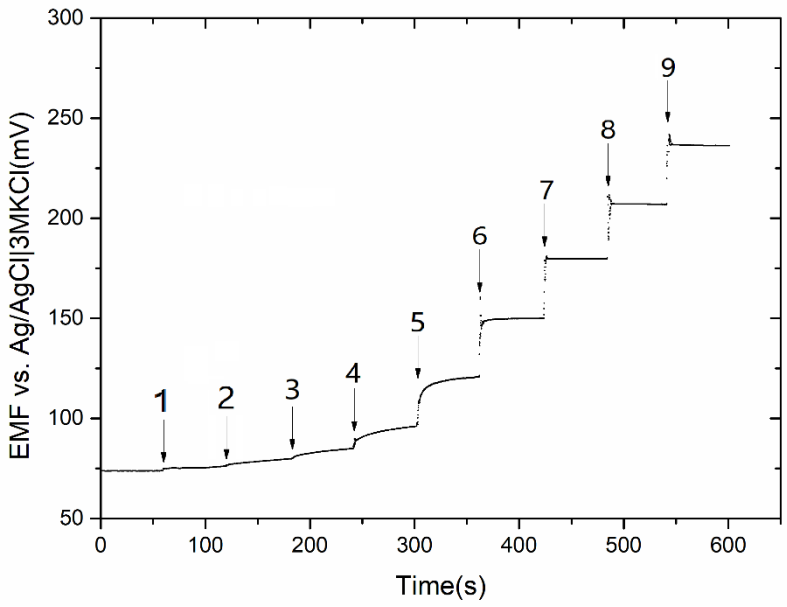

Figure 6. Dynamic response curve of ACh biosensor upon the addition of certain volumes of ACh stock solution to obtain various concentrations: (1) 10^{-7} M; (2) 3×10^{-7} M; (3) 10^{-6} M; (4) 3×10^{-6} M; (5) 10^{-5} M; (6) 3×10^{-5} M; (7) 10^{-4} M; (8) 3×10^{-4} M and (9) 10^{-3} M.

The calibration curve was plotted according to the dynamic response curve, as shown in Fig. 7. The slope in linear range of the ACh sensor was 58.3 mV/decade and the detection limit was 1.56 μM in the linear range of 10^{-5} M to 10^{-3} M. The slope was closer to Nernst theoretical value and the detection limit was lower than that in static detection. This may be due to the fact that the stirred ACSF increased the ACh activity and increased the contact between the ACh and the surface of the membrane, particularly in low concentrations.

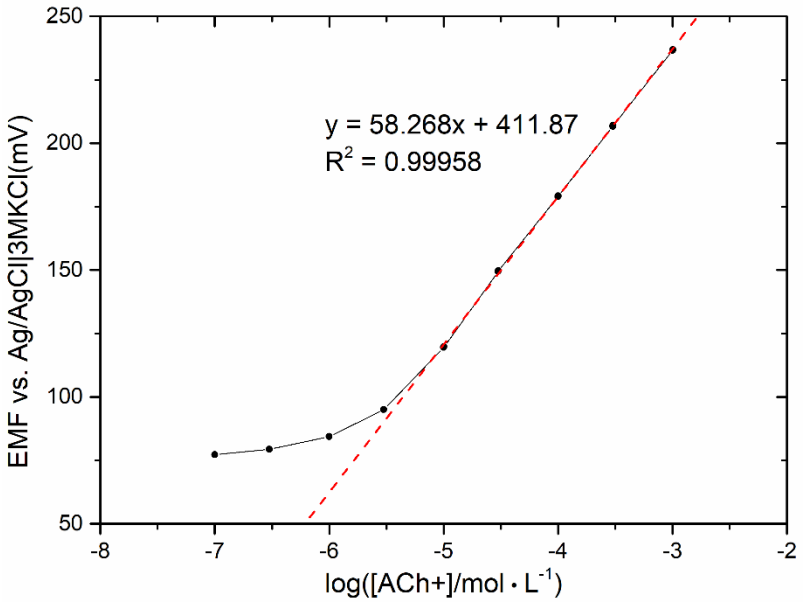

Figure 7. Calibration curve of the ACh biosensor in artificial cerebrospinal fluid.

4. CONCLUSIONS

A potentiometric biosensor for ACh detection was studied. The all-solid-state was realized by electropolymerizing a PEDOT/PSS layer on a gold disk as solid contact. Then the PEDOT/PSS layer was covered with an ACh selective membrane. The detection was minimally affected by variations of pH and other interferences, as tested in this paper. The experimental results also indicated that the ACh biosensor had satisfactory performances, such as a wide linear range, high sensitivity, good selectivity, repeatability and stability. The ACh biosensor could work properly in artificial cerebrospinal fluid. The technology is promising for fabricating ACh biosensors with different structures to satisfy the requirements of neurophysiological researches.

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