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Enhanced Measurement Stability and Selectivity for Choline and Acetylcholine by Capillary Electrophoresis with Electrochemical Detection at a Covalently Linked Enzyme-Modified Electrode

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Enzyme-modified microelectrodes were developed for the indirect amperometric detection of acetylcholine and choline following separation by capillary electrophoresis. Electrodes were prepared by first sequentially electrodepositing polypyrrole and polytyramine from the monomers on a 200-μm platinum electrode. The polymer bilayer provides enhanced selectivity and pendant amino groups for covalent coupling of acetylcholinesterase (AChE) and choline oxidase (ChO) by their reaction with glutaraldehyde. The AChE/ChO-modified electrode was then employed as an end-column detector to determine acetylcholine and choline with and without the internal standard butyrylcholine. Excellent operational stability during 2 days of continuous use was observed, with detection limits of 2 μM or 50 fmol for both acetylcholine and choline. The response from potential interferences, such as dopamine, catechol, and norepinephrine, were significantly reduced in comparison to a bare platinum electrode. The utility of this approach was demonstrated by monitoring the uptake of choline into synaptosomes.

Determination of the oscillating concentrations of acetylcholine (ACh) and its metabolic precursor, choline (Ch), is important in characterizing both normal and defective cholinergic neurotransmission. Such measurements are critical to research on neurodegenerative diseases that have been associated with impaired cholinergic function, such as Alzheimer's dementia and myasthenia gravis. Methods that can selectively and sensitively detect ACh and Ch concentrations are especially important in this regard.

Detection of ACh and Ch poses a significant analytical challenge because of the lack of a chromophore or electroactive center in both molecules. Thus, radiochemical, bioassay, or derivatization methods have been employed despite the challenges such procedures can present.[1] The complexity of biological fluids that characterize nerve tissue further hinders development of selective analysis procedures for ACh and Ch. Numerous electroactive and chromophoric chemical species coexist in these fluids and typically would present difficulties. However, indirect electrochemical detection offers one option for sensitive and selective determination of ACh and Ch. The basis of this method is the use of the selective enzymatic reaction of choline oxidase (ChO) to detect Ch or the use of acetylcholinesterase (AChE) with ChO to measure ACh. Both schemes produce hydrogen peroxide, which is then readily detected electrochemically. This strategy has been used to design microsensor devices for ACh or Ch,[2−10] which in some cases were used for in vivo measurements of Ch in rat brain.[7−10] Larger sensors for ACh and Ch have also been reported.[11−19] Selectivity is further enhanced in the standalone sensors by the use of a Nafion coating,[7−10,16] a nonconducting polymer[19] or cellulose acetate[6,11] membrane, an enzyme scavenger,[4,9] or a less positive detection potential afforded by a redox mediator.[2,4,7−10,14,16,17]

When a high degree of selectivity is necessary for the simultaneous determination of ACh and Ch, separation techniques such as high-performance liquid chromatography (HPLC) and gas

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chromatography (GC) have been used. These strategies were recently reviewed by Tsai.[20] One popular approach couples the enzyme reactions to HPLC followed by electrochemical detection of hydrogen peroxide derived from Ch oxidation. Potter et al.[21] demonstrated in 1983 that by introducing AChE and ChO into a postcolumn reactor with ACh and Ch after separation by HPLC, mass detection limits of 2 and 1 pmol were possible at a platinum electrode for ACh and Ch, respectively. Subsequent immobilization of AChE and ChO in a postcolumn reactor either by adsorption[22,23] or covalent attachment[24,25] significantly reduced the amount of enzyme needed and lowered detection limits to the fmol range.[25,26] Since these innovations, numerous studies have utilized a postcolumn reactor with a platinum working electrode to detect ACh and Ch.[27−35] In 1992, Vreeke et al.[36] reported the development of an amperometric sensor for hydrogen peroxide based on the immobilization of the enzyme horseradish peroxidase (HRP) and a redox polymer consisting of $[Os(bpy)_2Cl]^{3+/2+}$ coordinated to a polyvinylpyridine (PVP) backbone. When applied as the detector in the separation and detection scheme for ACh and Ch, the HRP−Os(PVP) electrode effectively mediates the electron transfer, allowing the use of a lower detection potential for hydrogen peroxide. Thus, detection of electroactive interferences is also significantly reduced.[37−40] An alternate approach that eliminates the postcolumn reactor by directly immobilizing the enzymes on an electrode surface for detection in HPLC[41−43] and flow injection methods[44,45] has been reported.

In a previous study, we prepared an AChE/ChO-modified microelectrode by chemically cross-linking the enzymes on the electrode surface with glutaraldehyde and used this system as a detector for measuring ACh and Ch after separation by capillary electrophoresis (CE).[46] A gradual loss in sensitivity with time was observed and was attributed to the instability of the enzyme layer under the hydrodynamic conditions at the end of the capillary. Similar difficulties have been reported using an AChE/ChO-based electrochemical detector with HPLC.[35,40] Nevertheless, baseline resolution and accurate quantitation of ACh and Ch with detection limits of 2 μM or 25 fmol were accomplished with the addition of butyrylcholine (BuCh) as an internal standard. To enhance the operational stability of the AChE/ChO microelectrode as a detector for CE, we have now developed an improved microelectrode detector system for CE that is based on the covalent attachment of the enzymes to the electrode surface. The preparation, characterization, and use of this electrode system for CE are described herein.

EXPERIMENTAL SECTION

Reagents. Acetylcholinesterase (EC 3.1.1.7, type III from electric eel, 1070 U/mg), choline oxidase (EC 1.1.3.17, *Alcaligenes* species, 12 U/mg), acetylcholine chloride (>99%), choline chloride (>98%), and butyrylcholine chloride (>98%) were purchased from Sigma (St. Louis, MO), stored in a desiccator at −10 °C, and weighed in a drybox when used. Choline was vacuum-dried overnight before use. *N*-Tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid (TES) (>99%), tyramine, catechol, dopamine, norepinephrine, and glutaraldehyde (grade I, 25% aqueous solution) were also purchased from Sigma. Platinum wire (200 μm in diameter, 99.99%) was obtained from Goodfellow (Berwyn, PA). Pyrrole (Aldrich) was distilled prior to use. All other chemicals were of analytical reagent grade and were used as received. Solutions were prepared with distilled deionized water purified to a resistivity of at least 17 MΩ-cm by a Barnstead B pure water purification system.

Apparatus. All routine electrochemical measurements, including the preparation of the enzyme-modified electrodes, were conducted in a conventional three-electrode electrochemical cell under anaerobic conditions with a Bioanalytical Systems (BAS, West Lafayette, IN) 100B electrochemical analyzer. A Ag/AgCl (3 M NaCl) (BAS, MF-2020) and a platinum wire were used as the reference and auxiliary electrodes, respectively. Deoxygenation was accomplished by bubbling argon through each solution.

Electrophoretic separations were conducted on a laboratory-built CE with an electrochemical (EC) detection system, which has been previously described.[47] For these experiments, an 88-cm fused-silica capillary with an i.d. of 50 μm and an o.d. of 360 μm was employed. Electrochemical detection also used a three-electrode configuration consisting of the enzyme-modified working electrode, a model RE−4 (BAS) Ag/AgCl reference electrode, and a platinum wire auxiliary electrode. The detection compartment was shielded in a Faraday cage to minimize contributions from external noise. The detection electrode was aligned in an end-column configuration[48] with the aid of a custom-made capillary−

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electrode holder (Allied Plastics, Inc., Toledo, OH).[49] An on-column fracture decoupler[50] was placed 2.5 cm from the capillary outlet and was used to isolate the detector from the separation voltage. Potential control and current monitoring were achieved with a BAS LC-4C amperometric detector, which was modified for use with CE. All data were collected by an IBM P166 MHz computer via an A/D converter and analyzed using P/ACE MDQ capillary electrophoresis system software (Beckman Instruments, Fullerton, CA).

Preparation of the Enzyme Detection Electrode. Platinum microdisk electrodes were prepared by a procedure similar to that used for carbon electrodes.[51] Briefly, a 2.5-cm length of 200-μm platinum wire was inserted into a 2-cm piece of fused-silica capillary (360-μm o.d., 245-μm i.d.) and sealed with epoxy glue (Borden Inc., Columbus, OH). An electrical connection was made at one end by attaching a piece of Cu wire using Ni print. At the other end, the tip was cut to form a microdisk surface. The microdisk was then polished to a smooth finish with a diamond suspension (1 μm, Buehler, Lake Bluff, IL), washed with distilled water, and sonicated in distilled water for 5 min.

The development of the immobilized enzyme detector for ACh and Ch was based on a method described by Palmisano et al.[52] The polished platinum electrode was first cycled between −0.21 and +1.19 V in 1 M H_2SO_4 until a steady-state voltammogram characteristic of a clean platinum surface was obtained.[53] The electrode was rinsed with distilled water and placed in a deoxygenated 0.1 M KCl solution containing 0.4 M pyrrole. A constant potential of +0.63 V was applied to induce formation of a polypyrrole (PPy) layer. Polymerization of pyrrole was continued until a charge density of ~320 mC/cm^2 was reached. The PPy-modified platinum electrode was then placed in a deoxygenated methanol solution containing 0.3 M NaOH and 0.1 M tyramine, and the potential was cycled between −0.01 and +1.6 V at 50 mV/s to deposit a layer of polytyramine (PTy) on top of the PPy. An amide linker for enzyme attachment was created by placing the Pt/PPy/PTy electrodes in a 2.5% (V/V) glutaraldehyde solution for 2 h at room temperature. After rinsing thoroughly with distilled water, the electrodes were transferred into pH 8.0 phosphate buffer containing 100 U/mL ChO and 167 U/mL AChE (ChO/AChE ratio was 1:1.6). After about 3 h, the enzyme electrodes were rinsed with phosphate buffer and stored in a desiccator at −10 °C until used.

Capillary Electrophoretic Separations. TES buffer (50 mM, pH 8.0) was used as the run buffer for all separations.[46,54] The applied voltage was 20 kV. Capillaries were conditioned before use with 1 M HCl (10 min), H_2O (10 min), and run buffer (30 min) at an applied pressure of 25 psi for each solution. Sample injection was accomplished by applying a pressure of 10 psi for 2 s, which corresponds to an injection volume of 25 nL. Electrochemical detection of hydrogen peroxide produced from the enzymatic reactions was carried out at +0.60 V vs Ag/AgCl. To prevent damage, the detection electrode was aligned after the capillary conditioning step. The capillary was rinsed and filled with water for overnight storage.

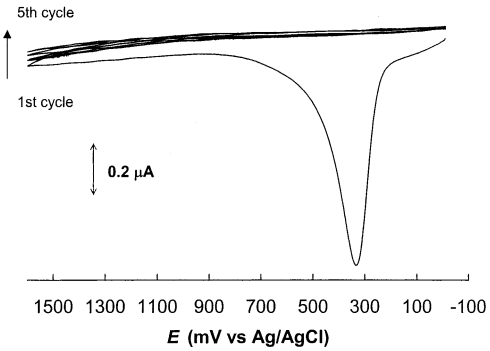

Figure 1. Cyclic voltammogram at the PPy-coated platinum electrode in a solution of 0.1 M tyramine in methanol containing 0.3 M NaOH. Initial potential, −0.010 V; scan rate, 50 mV/s.

Aqueous stock solutions of ACh and BuCh were made fresh daily, because they were subject to hydrolysis, but refrigerated Ch solutions were stable for at least one week. Calibration plots were obtained by plotting either the peak height for Ch or ACh or the ratio of the peak areas for Ch and ACh to the internal standard, BuCh (200 μM), versus analyte concentration. All measurements were performed in triplicate unless otherwise noted.

Preparation of synaptosomes and the measurement of Ch uptake into synaptosomes were performed as previously described.[46]

RESULTS AND DISCUSSION

Electrode Preparation. The AChE/ChO enzyme electrode was prepared by a multistep electrochemical and chemical modification procedure analogous to the method described by Palmisano et al.[52] for the development of a lactate biosensor. PPy and PTy were initially electrosynthesized to create a bilayer where the PPy layer functions as a permselective coating on the electrode, and the PTy layer completes the bilayer and provides pendant amino groups for covalent attachment of enzymes through a glutaraldehyde linker. On the basis of the charge accumulated (320 mC/cm^2) during PPy formation at the 200 μm platinum electrode used for this work, the film thickness was estimated to be 0.7 μm.[55] The cyclic voltammogram at the PPy-coated platinum electrode in 0.1 M tyramine between −0.01 and +1.60 V is shown in Figure 1. A single oxidation at +0.35 V is observed on the first scan, indicating the formation of PTy and subsequent loss of conductivity of the bilayer system as a result of overoxidation of the PPy layer, which is known to occur at potentials of approximately +0.7 V vs SCE or greater.[56,57] The overall loss of conductivity is reported to effectively terminate the polymerization reaction, creating a 50-nm-thick PTy layer[52,58] with pendant amino groups for covalent attachment of ChO and AChE.

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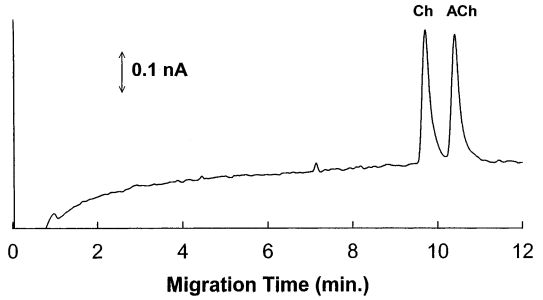

Figure 2. Electropherogram of a mixture of 100 μM ACh and Ch. Run buffer, 50 mM TES pH 8.0; separation voltage, +20 kV; detection potential, +0.60 V.

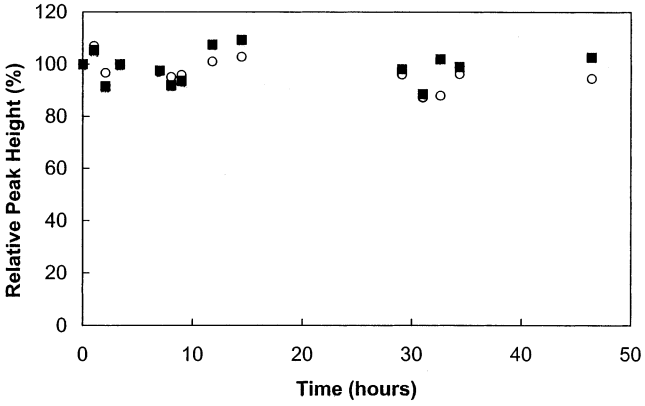

Figure 3. Normalized peak height of ACh (○) and Ch (■) as a function of time. Peak at time 0 is defined as 100%. Each point is the average response from three different electrodes to the injection of 100 μM ACh and Ch. Separation conditions are as described in Figure 2.

Enzyme solutions with ratios of ChO/AChE of 1:1.6, 1:1, 2:1, and 10:1 were evaluated. A ratio of ChO/AChE of 1:1.6 resulted in electrodes with the best responses for ACh and Ch and, therefore, was used to prepare the detector electrodes.

Electrode Characteristics and Stability. We have previously used an AChE/ChO electrode that was prepared by chemical cross-linking AChE and ChO onto a platinum microelectrode surface as a detector for CE.[46] This simple approach worked very well for the detection of ACh and Ch, but suffered from a gradual decay of the response with time as a result of the instability of the biocatalytic layer in a flowing system. Accurate quantitation in this case was achieved by incorporation of the internal standard BuCh, which is also a substrate for AChE. In principle, covalent attachment of the enzyme directly to the electrode surface through the PTy layer as described here should enhance the operational stability and lengthen the lifetime of the enzyme electrode.

Figure 2 illustrates a typical electropherogram for the separation of a standard mixture of 100 μM Ch (9.7 min) and ACh (10.4 min) with detection at an AChE/ChO electrode that was prepared by covalent attachment of the enzymes. ACh and Ch were readily separated in <11 min with a resolution of 0.95. The response reproducibility for the AChE/ChO electrode was examined by plotting the average relative peak heights for ACh and Ch versus time for three different electrodes. As is clearly observed in Figure 3, the response was essentially constant within experimental error, even after 48 h of continuous use in the detection cell containing 50 mM TES buffer (pH 8.0) at room temperature. A slight

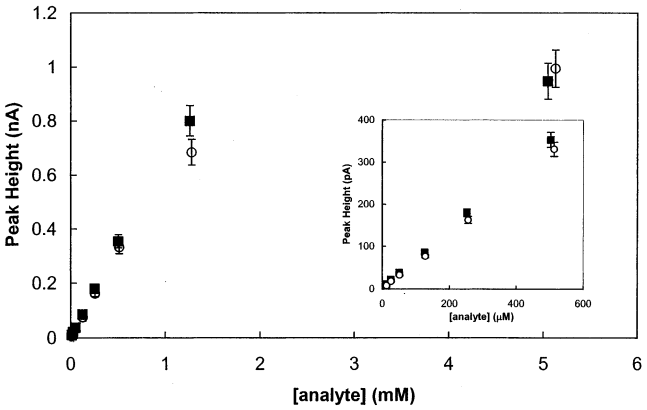

Figure 4. Calibration plots for ACh (○) and Ch (■) from 0 to 5 mM. Inset: Calibration plots from 0 to 500 μM. Separation conditions are as described in Figure 2.

decrease in response was observed after 2 days, most likely due to a small reduction in the activities of the bound enzymes. When electrodes were stored in a desiccator at −10 °C until use or between uses, the response remained constant for several months.

Performance Characteristics as a Detector for CE. The AChE/ChO electrode was examined over a range of ACh and Ch concentrations from 0 to 5000 μM (Figure 4). The response for both ACh and Ch between 0 and 500 μM (see Figure 4 inset) with any given electrode is linear; R^2 values for the data in the inset are 0.9995 and 0.9997 for ACh and Ch, respectively. Above 500 μM, the response exhibits negative deviations from linearity as the enzymes become saturated with substrate characteristic of Michaelis−Menten-type behavior.[10] The effective K_M values for the immobilized enzymes were determined to be 1.6 mM for ChO and 0.92 mM for AChE. These values are relatively close to those for the free enzymes,[59−63] indicating that immobilization has only minor effects on the enzyme kinetics and, thus, should not significantly affect quantitation of ACh and Ch by the CEEC method.

Electrode sensitivity, as measured by the slope of the calibration plot in the linear region, was determined for three different electrodes and found to vary from 0.63 to 6.4 pA/μM. This variation is attributed to the difficulty in controlling the amount and conformation of the enzymes during the enzyme loading step, even when identical preparation conditions are used. Thus, calibration of each electrode is required. Once calibrated, electrodes showed excellent reproducibility, with deviations in replicate measurements of <3% (n = 3). Limits of detection for ACh and Ch were determined to be 2 μM or 50 fmol at a signal-to-noise ratio of 3, which is consistent with our previous measurements by CEEC[46] and also at the level previously achieved by HPLC methods with a postcolumn reactor.[25,26]

BuCh was added as an internal standard to all standard solutions, and calibration plots were prepared by plotting the ratio of the peak height of either analyte to that of BuCh, which had a

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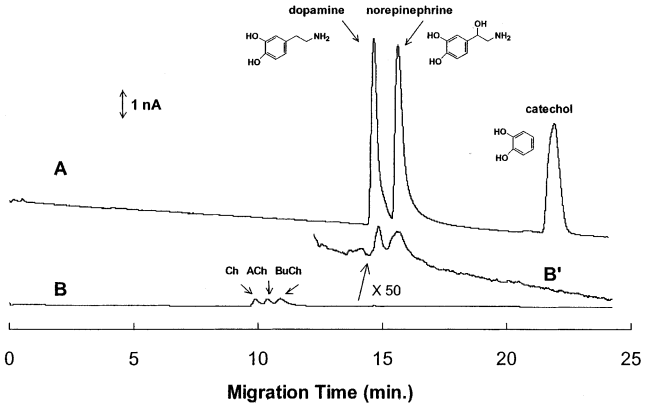

Figure 5. Electropherograms of a mixture of ACh (100 μM), Ch (100 μM), BuCh (200 μM), dopamine (50 μM), norepinephrine (50 μM), and catechol (50 μM). Detection at (A) an unmodified platinum electrode and (B) a covalently attached AChE/ChO-modified electrode. (B′) Magnification of B. Separation conditions are as described in Figure 2.

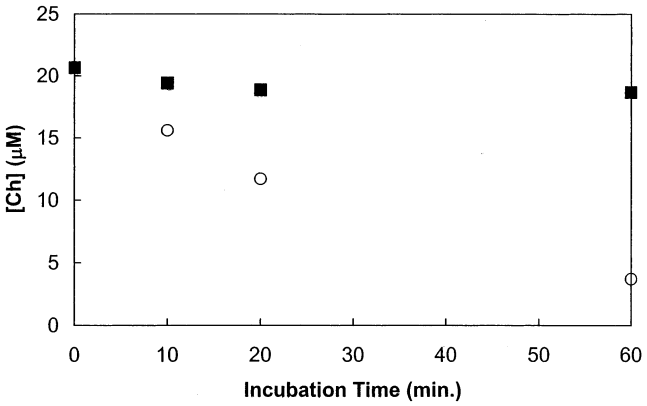

Figure 6. Plot of the change of Ch concentration versus incubation time in synaptosomes at 0 °C (■) and 37 °C (○). Synaptosome concentration, 3.9 mg/mL.

migration time of 11.5 min. Calibration plots with BuCh were very similar to those shown in Figure 4; however, the coefficient of variation (CV) in sensitivity between electrodes was <4% (n = 3). The detection limit with BuCh is also 2 μM, but it is better defined by the higher quality data obtained with the internal standard. For comparison, the CV for replicate measurements at each concentration of a calibration plot with BuCh is < 2% and < 7% without BuCh.

Selectivity. As noted earlier, many different approaches for enhancing selectivity have been incorporated into stand-alone sensors and separation methods with electrochemical detection for the determination of ACh and Ch. One of the most straightforward methods for enhancing selectivity for measurements in biological matrixes is the use of nonconducting polymer bilayers. Curulli et al.[19] and Palmisano et al.[52] utilized this approach in the development of choline and lactate sensors, respectively, for bulk solution measurements. The polymer structures readily reject interferences that would otherwise be detected at +0.70 V. We have used this approach to develop our enzyme microelectrode detector for the CE separation and detection of ACh and Ch and found similar selectivity advantages with the bilayer structure when used as a detector in a flowing system.

The selectivity of the AChE/ChO electrode for ACh and Ch was examined by CEEC in the presence of three representative and potentially important electroactive interferences in our biological experiments. The results for dopamine, catechol, and norepinephrine are shown in Figure 5. Upon injection of a mixture of Ch, ACh, BuCh, dopamine, catechol, and norepinephrine with a detection potential of +0.60 V at an unmodified Pt electrode (Figure 5A), large peaks due to dopamine, catechol, and norepinephrine were observed. The redox behavior of dopamine, catechol, and norepinephrine were also examined by cyclic voltammetry and square wave voltammetry under the same solution conditions. These compounds were oxidized at +0.14, +0.23, and +0.15 V, respectively and, thus, are easily detected when a platinum electrode is used as the detector. As expected, no response for Ch, ACh, or BuCh was observed in the absence of AChE and ChO. In contrast, when the AChE/ChO-modified PPy/PTy electrode was used (Figure 5B), Ch, ACh, and BuCh were detected; however, no response was observed for dopamine, catechol, or norepinephrine at the same sensitivity used for Figure 5A. Peaks for dopamine and norepinephrine were visible only when the sensitivity was increased by 50-fold (part B′ of Figure 5′). A peak for catechol was not observed,even when further magnified. This result clearly demonstrates the advantage gained by using PPy and PTy as permselective layers on the platinum electrode surface. The polymer films selectively reject larger molecules, whereas hydrogen peroxide permeates the bilayer and is oxidized at the platinum electrode. Moreover, oxidation of interferences on the film surface is minimized, because the nonconductive PPy and PTy layers inhibit electron transfer through the bilayer.

A commonly reported method for ACh and Ch involves HPLC separation and the use of a postcolumn enzyme reactor to generate hydrogen peroxide, which is detected at a variety of electrode types. One innovation of note is the incorporation of a third enzyme, horseradish peroxidase, onto a conducting polymer backbone to catalyze the reduction of hydrogen peroxide at the electrode surface.[37−40] This effectively enhances the selectivity by permitting the application of a detection potential that is lower than the oxidation potentials needed to detect electroactive interferences. In comparison to these previous studies, the PPy/PTy bilayer provides a simpler approach to enhance selectivity through the rejection of interferences. At the same time, the need for the postcolumn enzyme reactor is eliminated, since the enzymes are covalently attached directly to electrode.

Application to Monitoring Choline Uptake in Synaptosomal Preparations. One of our goals for developing a selective and sensitive method for detecting ACh and Ch is to evaluate Ch uptake through the high affinity choline uptake transport channel under conditions of selective inhibition and subsequent inactivation by a series of well-established cholinotoxic agents.[64−69] For the purpose of demonstrating the feasibility of CE with the

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covalently linked enzyme electrode as a method to monitor Ch uptake, freshly prepared synaptosomes were used as a model for Ch transport. Figure 6 describes the kinetics of Ch uptake by synaptosomal suspensions at 0 and 37 °C by monitoring the decrease in Ch concentration in the extracellular solution. This experiment defines the baseline response for Ch uptake into synaptosomes at the physiological temperature of 37 °C where normal Ch transport is assumed to occur and at 0 °C where Ch transport is assumed to occur to a negligible extent. It is easily seen from Figure 6 that the relative Ch concentration ($n = 2$) after 20 min at 0 and 37 °C are 0.91 and 0.57, respectively, and 0.90 and 0.18 after 60 min, indicating significant transport of Ch into the synaptosomes at 37 °C. This result is consistent with our previous results using an AChE/ChO enzyme electrode as a detector for CE prepared by chemical cross-linking[46] and a radiochemical assay by Patel et al.[64] Any apparent decrease in Ch concentration at 0 °C is most likely due to other mechanisms, such as diffusion. By comparing the data obtained at 0 and 37 °C, the rate of Ch transport through the high affinity choline uptake channel is estimated to be ~0.1 μM/min for every mg/mL of synaptosome.

CONCLUSIONS

An enzyme-modified microelectrode sensor was prepared by the covalent attachment of AChE and ChO to an electrosynthesized PPy/PTy bilayer coating and used for the indirect electrochemical detection of ACh and Ch following separation by CE. Improved operational stability was observed relative to our previous work as a result of the direct attachment of the enzymes to the pendant amino groups of PTy by reaction with glutaraldehyde. Therefore, the use of an internal standard is not required to minimize the effect of a decaying electrode response. The overall selectivity of this approach is also improved and is achieved by a combination of selectivity at three levels: the high efficiency of the separation system, the selectivity of the enzyme reactions, and the permselectivity of the polymer layers. The PPy/PTy bilayer is shown to dramatically reduce the response to potential interferences, such as dopamine, catechol, and norepinephrine. The procedure for detector development described here is relatively simple to implement and offers many of the same advantages for ACh and Ch measurements as other more complicated electrochemical strategies. Furthermore, the combination of miniature size, operational stability, and selectivity are important advances for analysis of ACh and Ch in biological assays by CEEC.

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