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Electrocatalytic Microelectrode Detectors for Choline and Acetylcholine following Separation by Capillary Electrophoresis

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Abstract

Two electrocatalytic enzyme modified microelectrode systems were employed as end-column amperometric detectors of choline (Ch) and acetylcholine (ACh) following separation by capillary electrophoresis (CE). Horseradish peroxidase crosslinked in an Os based redox polymer hydrogel (HRP-Os) was physically adsorbed on Au microelectrodes followed by chemical crosslinking of the enzymes acetylcholinesterase (AChE) and choline oxidase (ChO). An alternative approach utilized the deposition of the transition metal catalyst, Prussian Blue (PB), on Pt microelectrodes as the electrocatalyst. Utilizing butyrylcholine (BuCh) as an internal standard, the HRP-Os/AChE-ChO and PB/AChE-ChO electrodes exhibited excellent linear responses from 2–2000 μM and 10–2000 μM , respectively, for both Ch and ACh. Detection limits of 0.1 μM or 38 amol were determined for the HRP-Os/AChE-ChO electrode. The limit of detection for ACh and Ch at the PB/AChE-ChO electrode was 5 μM or 9.5 fmol. The electrodes were operated at potentials of +0.10 and –0.10 V vs. Ag/AgCl (3M NaCl), respectively, and thus minimized the potential response from oxidizable interferences. In addition, both electrocatalytic electrodes showed good operational stability for more than 70 hours. The enhanced detection capability of the HRP-Os/AChE-ChO and PB/AChE-ChO electrodes in combination with efficient CE separation of Ch and ACh provides a new sensitive and selective strategy for monitoring and quantifying these cholinergic biomarkers in biological fluids.

Introduction

Neurological disorders such as Alzheimer's disease and related dementia are correlated with physical symptoms of early cognitive loss followed by more severe dementia. Although Alzheimer's disease presents a complex pathology that is still under intense investigation, significant evidence points toward the depletion of presynaptic cholinergic markers during various stages of the disease.^{1–5} Understanding the subtle chemical changes that occur in the cholinergic system and how these changes affect neuronal degradation is a challenging analytical problem, yet critical to advance predictive and diagnostic tools or treatment strategies.

Fundamental to the investigation of changes in the cholinergic system is the detection of the small molecule cholinergic biomarkers, choline (Ch) and acetylcholine (ACh). This task is even more difficult because these molecules lack a chromophore or fluorophore, and are not electroactive. Therefore, most analytical methods to detect Ch and ACh historically utilized radiochemical, bioassay or derivatization methods.⁶ Further challenges arise from the complicated sample matrix associated with biological fluids containing low levels of Ch and ACh. Consequently, efficient and rapid separation strategies in combination with sensitive detection methods are highly desirable. Separation of Ch and ACh has been addressed by the

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application of chromatographic⁷ and more recently, capillary electrophoresis (CE) methods.⁸ When coupled to mass spectrometry^{9–14} and electrochemical (EC)⁸ detection methods, femtomol to attomol mass detection limits were achieved.

One of the goals of our research is to develop quantitative non-radiochemical methods to evaluate Ch transport through the high-affinity Ch uptake transporter protein (CHT). In particular, we are interested in methods that permit discrimination of subtle changes in Ch concentrations under conditions of presynaptic degradation induced by a series of inhibitors of CHT. The ability to accurately assess such small fluctuations may lead to protocols for early detection of neurodegenerative conditions. To accomplish this goal, CE with indirect EC detection using enzyme-based microelectrodes was employed.⁸ CE offers the advantage of small sample volumes, high separation efficiency, use of aqueous solvents and a relatively short analysis time, while EC detection offers excellent selectivity and sensitivity and the ability to modify microelectrodes to gain further selectivity for a targeted analysis. Additional selectivity for biological samples is also achieved by the application of microdialysis sampling techniques prior to the analysis step. As a result of these features, CE methodologies have emerged as important tools for neurobiological investigations.¹⁵

EC methods for the indirect detection of Ch and ACh utilize enzyme modified electrodes. This approach has been very popular and is based on the catalytic hydrolysis of Ch by choline oxidase (ChO) or ACh by a combination of acetylcholinesterase (AChE) with ChO. Both enzymatic reaction schemes generate hydrogen peroxide which is then oxidized or reduced at an electrode surface. Various enzyme modified macro- and microelectrode designs and their applications for Ch and ACh detection were summarized in a recent review.⁸ For our studies, we employed a 25 μ M platinum wire that was coated with ChO and AChE by chemically crosslinking the enzymes and physically adsorbing them to the electrode surface. Detection limits of 100 amol for Ch and 1 fmol for ACh were possible using this electrode as the detector for CE coupled with an internal standard method.^{8,16} This approach was subsequently used for evaluation of Ch transport rates in the presence of a new class of quaternary ammonium alkyl-substituted inhibitors of CHT.^{17–19} By varying the efficacy of the inhibitor, differences in Ch transport rates and quantitative evaluation of their potency were determined. An alternative detector design was developed for this purpose by covalent attachment of the enzymes to a platinum electrode.²⁰ This design yielded greater operational stability and eliminated the need for an internal standard, but resulted in a more extensive fabrication procedure with no significant improvement in detection limits.

An approach to further improve the electrochemical detection of low endogenous concentrations of Ch and ACh is to enhance the analytical response through electrocatalysis. Two electrocatalytic systems in particular that are known to efficiently catalyze the reduction of hydrogen peroxide are attractive for this purpose. One system is based on the co-immobilization of horseradish peroxidase (HRP) and a redox polymer of $[\text{Os}(\text{bpy})_2\text{Cl}_2]^{3+/2+}$ coordinated to a polyvinyl pyridine (PVP) backbone. In 1992 Vreeke et al. were the first to use this approach for the amperometric detection of hydrogen peroxide.²¹ Subsequently Garguilo et al. developed an amperometric sensor for Ch and ACh by co-immobilizing HRP, ChO and AChE with the crosslinked redox polymer onto carbon electrodes.^{22,23} A similar strategy was employed to develop a multienzyme electrode detector for ACh detection in combination with flow injection methods.^{24,25}

The second electrocatalytic system utilized Prussian Blue (PB) or ferric ferricyanide, $\text{Fe}_4^{\text{III}}[\text{Fe}^{\text{II}}(\text{CN})_6]_3$, as the electrocatalyst. PB is a well known coordination compound that is formed from an aqueous solution of FeCl_3 and $\text{K}_3\text{Fe}(\text{CN})_6$ either spontaneously or under the influence of a constant potential.^{26,27} PB undergoes a four-electron, K^+ dependent reduction

detector, which was modified for use with CE, was used to apply the detection voltage and monitor the resulting current. The modified microelectrodes were carefully aligned with the capillary outlet to optimize contact with the capillary flow and minimize disruption of the electrocatalytic layer through contact with the sides of the capillary. A 145 cm polyimide coated fused-silica capillary with an i.d. of 50 μM and an o.d. of 300 μM (Polymicro Technologies, Phoenix, AZ) was used for separation of the analytes. The decoupler was placed ~ 2.5 cm from the capillary outlet. Electropherograms were generated by applying separation voltages from 9–20 kV using a Spellman CZ100R high-voltage power supply (Spellman, Plainview, NY). The separation current during operation ranged from 4 to 20 μA . Data were collected by an IBM P166 MHz computer through an A/D converter. P/ACE MDQ Capillary Electrophoresis System software (Beckman Scientific Instruments, Fullerton, CA) was used for data analysis.

Electrochemical measurements were conducted to evaluate the electrode preparation procedures using a BAS 100B electrochemical analyzer interfaced to a Gateway P5–120 computer. A conventional three electrode configuration was used with either a modified or unmodified working microelectrode, a spiral platinum wire auxiliary electrode and a Ag/AgCl (3 M NaCl) reference electrode. The reference electrode was stored in aqueous 3 M NaCl when not in use. An argon purge was used to deoxygenate all solutions prior to the bulk electrochemical measurements.

Methods

Preparation of Au and Pt Microelectrodes

Au and Pt microelectrodes were prepared as previously described.¹⁶ After fabrication, the bare microelectrode tip was rinsed with acetone, deionized water and finally with acetone. The electrodes were dried in air at ambient temperature for 10 minutes before performing the immobilization procedure. Au microelectrodes were used as the substrate for the HRP-Os/AChE-ChO electrode and Pt microelectrodes were used for the PB/AChE-ChO electrode.

Preparation of HRP-Os/AChE-ChO Modified Au Electrodes

The Au microelectrode tip was bent into a loop and 0.5 μL of the HRP-polymer solution was carefully placed on the loop. The electrode was dried in air for 30 minutes in an inverted position at ambient temperature. This immobilization step was repeated twice. The resultant electrodes were then gently dipped into a 7 μL aqueous solution containing 6 units of ChO, 25 units of AChE and 0.5 μL of a 25% aqueous glutaraldehyde solution. The enzymes were physically adsorbed onto the electrode surface as crosslinking occurred, and then allowed to dry in air for 1 hour before use. This solution was used to coat 3–4 additional electrodes.

Preparation of PB/AChE-ChO Modified Pt Electrodes

PB films were prepared on a Pt electrode surface by both an electrochemical deposition method and a slow film formation method. Electrodes from both methods were evaluated by comparison of the resulting electrochemical properties. PB films formed by electrodeposition yielded a more reproducible film and thus were used for the CE studies.

Electrochemical Deposition Method

PB films were electrochemically deposited on Pt microelectrodes following the general procedure of Karyakin et al. with some modifications.^{26, 30} Various concentrations ranging from 2 to 50 mM of a 1:1 molar ratio of $\text{K}_3[\text{Fe}(\text{CN})_6]/\text{FeCl}_3$ were prepared in a 0.1 M HCl/KCl aqueous solution. PB was deposited at a constant potential of + 0.4 V for 180 s. The resultant PB film was activated by cycling between -0.50 to $+0.35$ V at 40 mV/s in a 0.1 M HCl/KCl solution until a stable voltammogram was obtained (approximately 20 sweeps, 10

cycles). The electrodes were thoroughly washed with deionized water and then modified with a 7 μ L aqueous solution containing 6 units of ChO, 25 units of AChE and 0.5 μ L of a 25% aqueous glutaraldehyde solution. The electrodes were stored in the dark at -20°C .

Slow Film Formation Method

Neff demonstrated a method of preparing PB films by simply immersing a cathodized Pt electrode in a solution of ferric ferricyanide.³⁹ An analogous method was employed where a 1:1 molar ratio of various concentrations (4 to 25 mM) of $\text{K}_3[\text{Fe}(\text{CN})_6]/\text{FeCl}_3$ in 0.1 M HCl/KCl were used. Pt microelectrodes were then dipped into an unstirred solution and PB films were allowed to grow for ~ 72 h in the dark. The electrodes were then washed with deionized water and cured in an oven at 80 – 100°C for ~ 12 h. The resultant PB film was activated by repeated cycling between -0.50 to $+0.35$ V at 40 mV/s in a 0.1 M HCl/KCl solution until a stable voltammogram was obtained.^{30,40} Further modification with AChE and ChO by chemical crosslinking with glutaraldehyde was performed as described above.

Capillary Electrophoresis

Separation conditions were optimized by variation of the run buffer, either TES (50 mM, varying pH) or phosphate buffer pH (20 mM, pH 7.0), the column length (85–145 cm) and separation voltages (9 to 20 kV). Prior to use, capillaries were conditioned with HCl (10 min, 25 psi) to suppress electroosmotic flow, followed by H_2O (10 min, 25 psi), and finally rinsed with TES (30 min, 25 psi). Samples were injected by semi-automatic pressure injection using high purity argon at 5–25 psi for 0.1–5 s corresponding to an injection volume of 0.4–95 nL. After each run the capillary column was washed with run buffer at 5 psi for 4 minutes. When not in use, the capillary was rinsed and filled with water. To prevent denaturing of the enzymes and damage to the detector electrode, the electrode was aligned to the capillary after conditioning the column. All standard solutions of Ch, ACh and BuCh were prepared fresh daily in deionized water and stored in ice.

The optimum operating potentials for the HRP-Os/AChE-ChO and PB/AChE-ChO electrodes were determined by injecting a standard Ch solution and monitoring the current response as a function of potential. The Au and Pt microelectrodes were subsequently operated at $+0.10$ V and -0.10 V vs. Ag/AgCl (3 M NaCl), respectively.

The linear ranges for detection of Ch and ACh were determined at the HRP-Os/AChE-ChO modified Au electrode (2 to 2000 μM) and the PB/AChE-ChO modified Pt electrode (10 to 2000 μM) using the concentration range noted. The concentration of BuCh was 200 μM for all analyses. The response at each concentration of Ch and ACh was recorded as the average of triplicate measurements. The operational stability of the modified electrodes was determined by repetitive injections of a standard stock solution of Ch, ACh and BuCh in a 1:1:1 ratio corresponding to a final concentration of 500 μM . Electropherograms were continuously collected for the first 24 h and then for an 8 h time block every 12–15 h until the electrode performance severely deteriorated.

Results and Discussion

Microelectrode Detector Design and Characterization

A schematic representation of the electrode design and operation is shown in Figure 1. Electrocatalytic electrodes with the best performance as a detector for CE were achieved using a sequential two-step layer-by-layer procedure where the electrocatalytic layer was deposited directly on the electrode surface followed by physical adsorption of AChE and ChO. This provided stable integration of AChE and ChO onto the electrocatalytic layer. Each method was evaluated for their electrocatalytic activity, which was estimated from the response observed

from cyclic voltammetry measurements and by the detection of Ch following electrode modification with ChO and AChE.

HRP-Os/AChE-ChO Au electrode

The sequential layer-by-layer deposition strategy was especially important for the HRP-Os/AChE-ChO electrode because the simultaneous deposition of the three enzymes through chemical cross-linking with glutaraldehyde resulted in loss of catalytic activity of the HRP-Os polymer and an electrode with limited functionality. Partial loss of electrochemical activity from the Os (III/II) redox couple was also reported for the cross-linked polymer in a similar enzyme electrode preparation approach using polyethyleneglycol diglycidyl ether (PEG) as the cross-linking agent.²² Although the reason for this loss of electrocatalytic activity was not clear, simultaneous deposition presumably disrupts the 3-dimensional unit of the redox hydrogel through extensive enzyme cross-linking with glutaraldehyde. The observation of residual electrochemical activity with PEG suggests the longer PEG chain was more effective in maintaining the electron-transfer properties of the HRP-Os electrocatalyst. The sequential deposition strategy used here more closely parallels the approach used by Larsson et al.²⁵ where HRP, ChO, AChE and an Os-polyvinyl imidazole polymer were successfully co-immobilized by crosslinking with PEG. This enzyme electrode showed very good response and was used for determination of ACh levels in brain microdialysate.

The HRP-Os/AChE-ChO electrode was evaluated at carbon, platinum and gold microelectrode surfaces. The metal electrodes provided superior durability when compared to carbon microelectrodes. The redox cycling capability of the Os(III/II) redox couple in the HRP-Os electrocatalytic layer is clearly shown in Figures 2a and 2b. Under the optimized deposition conditions at a Pt microelectrode substrate the reversible Os(III/II) redox couple was observed with $E^0 = +0.389$ V. Addition of a layer of AChE and ChO resulted in a similar voltammogram with $E^0 = +0.400$ V. However, the Pt-based HRP-Os/AChE-ChO electrode performed poorly as a detector for CE. Switching the substrate to Au greatly improved the detector performance and was therefore used for all CE measurements. The improved performance of the Au-based electrode was due to the greater affinity of the HRP layer to the Au surface, which provided a consistent electrocatalytic layer for the deposition of AChE and ChO. The Au electrodes were stable in the hydrodynamic conditions of the capillary and could be operated at potentials between -0.200 and $+0.400$ V. The electrodes were operated at constant potential of $+0.10$ V, which is 0.5 V less positive than the potential used for the AChE-ChO Pt electrode.¹⁶

PB/AChE-ChO Modified Pt Electrodes

Two different literature strategies were modified for the deposition of PB films: slow film formation³⁹ and electrodeposition.^{26,30} A comparison of the cyclic voltammograms of the films for each method is presented in Figure 3.

The basis of the slow film formation method involved placing the Pt microelectrode substrate in direct contact with a stoichiometric solution of $K_3[Fe(CN)_6]$ and $FeCl_3$. The PB films were allowed to grow on the Pt surface undisturbed until a film of sufficient thickness was obtained. 0.1 M HCl/KCl solutions with 4, 8, 15 and 25 mM of 1:1 $K_3[Fe(CN)_6]/FeCl_3$ were evaluated. Film thickness was proportional to the solution concentration and was easily distinguished by visual inspection. At concentrations greater than 8 mM, thick amorphous particles formed on the electrode surface. Although thicker films were formed at 8 mM, the greatest current response was obtained after activation by potential cycling with films prepared in the 4 mM $K_3[Fe(CN)_6]/FeCl_3$ solution. Films grown in the higher concentration solutions exhibited similar current responses but greater peak separations in the cyclic voltammograms.

Under the influence of a constant potential, PB films were spontaneously electrodeposited onto the Pt microelectrodes by the reduction of ferric ions in the presence of ferricyanide. Electrodeposition is a convenient method for film formation because the amount of PB catalyst deposited, film thickness, and the PB electrochemical properties can be readily controlled by adjusting simple parameters such as the deposition potential, deposition time and type of electrode material.

In order to determine the optimum concentration of $K_3[Fe(CN)_6]/FeCl_3$ for the deposition, various concentrations ranging from 2 to 50 mM were evaluated. Similar to the slow film formation method a concentration of 4 mM provided the highest current response in the cyclic voltammograms of the resultant films. A deposition time of 180 s was selected as the optimum time to produce the greatest current response in the cyclic voltammogram of the PB film. The average charge for film formation was determined to be $40.2 \pm 4.3 \mu C$ ($n = 6$). Assuming 4 electrons are transferred, the total amount of PB deposited was 104.2 pmoles. Since the exact surface area of the microelectrode was unknown, the thickness of the deposited film was estimated to be 1.33 nmol/mm^2 for a 1 mm long cylindrical microelectrode. The quality of the resultant PB film also can be judged by the sharpness of the oxidation and reduction peaks after activation by redox cycling. At very high concentrations, the cathodic and anodic peaks were broad with large peak separations. More symmetrical voltammograms were observed at lower concentrations, indicating facile reversible reduction and oxidation of the PB catalyst under such conditions.

PB films developed from slow film formation and electrodeposition processes yielded comparable results (Figure 3). PB films formed by electrodeposition were used for further studies because of their ease of preparation and more reproducible film characteristics.

Separation and Detection of Ch, ACh and BuCh

Baseline resolution of Ch, ACh and BuCh was previously achieved with a AChE-ChO Pt microelectrode detector and an 80 cm capillary with 50 mM TES buffer pH 8.¹⁶ With these experimental conditions, baseline resolution was difficult to achieve with the electrocatalytic detectors due to broadening of the peaks. The addition of the electrocatalyst increases the diffusion distance in the bilayer system and is likely responsible for the increased tailing of the peaks for each analyte. Optimization of the buffer conditions was attempted using an 80 cm silica capillary without success even at low pH where the electroosmotic flow of the system was expected to be smaller. However, lengthening the capillary to 145 cm in combination with a 50 mM TES pH 8.0 run buffer successfully resulted in baseline separation with both electrode systems. The electropherograms for the separation and detection of Ch, ACh and BuCh at the HRP-Os/AChE-ChO Au and PB/AChE-ChO Pt microelectrodes are shown in Figures 4a and 4b, respectively. The peak area for the PB/AChE-ChO system was comparatively broader than for the HRP-Os/AChE-ChO detector. Since the amperometric response of the detector is directly related to the rate of electron transfer to the electrode surface, a broader signal for the PB/AChE-ChO system may be attributed to slower catalytic conversion of H_2O_2 by PB.

Linear Range

The linear range for the electrocatalytic microelectrodes was evaluated by plotting the ratio of the peak areas for Ch and ACh solutions of varying concentration to that of the internal standard, BuCh. Calibration plots for each electrode and analyte were generated and analyzed for both detector systems. AChE and ChO obey Michaelis-Menten kinetics and therefore, large injection volumes resulted in saturation of the current response and deviation from linearity, especially at higher analyte concentrations. To minimize this effect an injection volume of 0.8 nL was employed for experiments with the HRP-Os/AChE-ChO Au microelectrode. A linear response was then obtained from 2 to 2000 μM for both Ch and ACh. Three different electrodes

were examined and R^2 values ranged from 0.9974 to 0.9995 for Ch and from 0.9957 to 0.9993 for ACh. The linear range for the PB/AChE-ChO Pt detector was found to span from 10 to 2000 μM . A larger injection volume of 38 nL was used in this case due to the lower sensitivity of this electrode system. The R^2 values for Ch ranged from 0.9941 to 0.9996, while that of ACh ranged from 0.9950 to 0.9996. Concentrations beyond 2000 μM were not examined, as very high concentrations caused overloading of the column and inefficient separations of the analytes. Since in vivo concentrations of these cholinergic analytes are in the sub-micromolar to micromolar range, studies at lower concentration ranges are more relevant for further applications.

The sensitivity of the electrodes was determined from the slope of the calibration plots for each electrode system using three different electrodes. The average sensitivity was 0.0069 ± 0.0018 units/ μM for Ch and 0.0072 ± 0.0015 units/ μM for ACh at the HRP-Os/AChE-ChO Au detector. The response of the PB/AChE-ChO Pt detector to Ch yielded an average sensitivity of 0.0053 ± 0.0022 units/ μM for Ch and 0.0051 ± 0.0020 units/ μM for ACh, where a unit is defined as the change in the ratio of the peak of Ch/BuCh or ACh/BuCh. The slight variation in sensitivity of the electrocatalytic electrodes was expected due to the difficulty exactly reproducing the fabrication procedure, especially the amount of AChE and ChO adsorbed on the electrode surface and the alignment with the capillary outlet.

Limit of Detection

A detection limit of 0.1 μM for Ch and ACh was achieved using the HRP-Os/AChE-ChO Au detector. Figure 5 shows the electropherogram obtained from the injection of a 0.1 μM solution of Ch, ACh and BuCh at 5 psi for 0.1 s, which corresponds to an injection volume of 0.38 nL. Thus, a mass detection limit of 38 amol ($S/N = 3$) for both ACh and Ch was determined, which is approximately a factor of 25 improvement for ACh and a factor of 3 for Ch compared to our previous method using the AChE-ChO Pt microelectrode: 100 nM (1 fmol) for ACh and 10 nM (100 amol) for Ch.^{8,16} The limit of detection for ACh and Ch at the PB/AChE-ChO electrode was slightly higher at 5 μM or 9.5 fmol.

Operational Stability of the Electrodes

One of the challenges of using enzyme modified microelectrodes for CE is that the electrodes are operated under hydrodynamic conditions where gradual loss of enzymes from the surface leads to decreased sensitivity. The electrocatalytic electrodes showed good performance for more than 40 runs when used during a period of 72 hours. Since samples were injected using a semiautomatic method and extremely small sample volumes, the absolute peak area of the analytes could not be used as a standard for evaluating the operational stability of the electrodes. For the HRP-Os/AChE-ChO system there was an average decrease in the peak area for Ch by 8% during the first 10 hours of operation, which dramatically decreased to 48% after 24 hours of continuous usage. The corresponding decrease in ACh peak area was 4% and 47%, respectively. The PB/AChE-ChO microelectrode showed an average drop of 14% for both Ch and ACh after 10 hours of operation, which further decreased to 46% and 40% for Ch and ACh after 24 hours of usage.

The use of BuCh as the internal standard corrects for any fluctuations in signal with time and permits accurate quantitation with the electrocatalytic detectors. Therefore, a stable response was observed for each electrode for both Ch and ACh for extended periods of time. The internal standard approach allows the use of the simpler electrode preparation procedure and avoids the need to covalently link the enzymes to the electrode surface.²⁰

Figures 6 and 7 illustrate the operational stability of the HRP-Os/AChE-ChO and PB/AChE-ChO microelectrodes as a function of time and number of runs. In both cases the response is

quite stable for 72 hours ($n = 11$) and for as many as 88 runs for the HRP-Os/AChE-ChO and 62 runs for the PB/AChE-ChO electrode. Each point in Figure 6 represents the average peak area ratio from three different electrodes normalized to the initial peak ratio as a function of time. The average normalized response and standard deviation for the HRP-Os/AChE-ChO detector was 1.04 ± 0.03 for Ch and 1.11 ± 0.05 for ACh, and for the PB/AChE-ChO detector was 1.01 ± 0.03 for Ch and 1.01 ± 0.02 for ACh. For the data in Figure 7, the average response and standard deviation for the HRP-Os/AChE-ChO detector was 1.25 ± 0.05 for Ch and 1.18 ± 0.04 for ACh, and for the PB/AChE-ChO detector was 1.62 ± 0.08 for Ch and 1.22 ± 0.06 for ACh.

Conclusion

Two electrocatalytic microelectrode detector systems for the sensitive detection of ACh and Ch following CE separation were developed and evaluated. The electrodes were designed with a microelectrode substrate upon which sequential layers of either a HRP-Os hydrogel or PB electrocatalyst, and an enzyme film of AChE and ChO were deposited. The layer-by-layer approach resulted in sensitive and selective detection systems with linear ranges over three orders of magnitude with fmol to amol mass detection limits. These detection limits rival or exceed our previously reported detection limits using a AChE-ChO Pt electrode following CE separation^{8,16} Given the AChE-ChO Pt electrode readily allowed detection of changes in Ch concentrations in microdialysis samples from the extracellular fluid of brains of awake, freely moving rats,⁸ the electrocatalytic detectors should find similar utility for the determination of low endogenous levels of cholinergic biomarkers. For comparison, the tri-enzyme HRP-Os/AChE-ChO bilayer electrode detection system has previously seen only limited use for flow injection analysis of ACh in extracellular brain samples.^{24,25} In these cases, detection limits for ACh of 4.8 nM (~ 10 fmol)²⁴ and 0.3 μ M (0.4–15 pmol)²⁵ were reported. The lower mass detection limits for CE clearly demonstrates the advantage of the small sample volumes coupled to the exceptional sensitivity of an electrocatalytic enzyme electrode. The HRP-Os/AChE-ChO and PB/AChE-ChO also can be operated at potentials at least 0.5 V less positive than the AChE-ChO Pt electrode to avoid common oxidizable biological interferences. Therefore, electrochemical detection with the electrocatalytic electrodes coupled with CE yields multiple levels of selectivity enhancement for the efficient separation and sensitive detection of Ch, ACh and BuCh.

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Figure 1.
ACh and Ch detection scheme for the bilayer electrocatalytic enzyme electrodes.

Figure 2a **Figure 2b**

Figure 2. Comparative cyclic voltammograms for (a) a bare Pt microelectrode and Pt microelectrode modified with the HRP-Os redox polymer and (b) a Pt microelectrode modified with only ChO and AChE and the HRP-Os/AChE-ChO modified electrode system. Solution conditions: 20 mM phosphate buffer pH 7.0. Scan rate: 20 mV/s. Arrows indicate direction of scan.

Figure 3. Comparative cyclic voltammograms for PB modified Pt microelectrodes prepared from a 4 mM solution of $K_3[Fe(CN)_6]/FeCl_3$ in 0.1 M HCl/KCl by electrodeposition and slow thermal deposition methods. Solution conditions: 0.1 M HCl/KCl Scan rate: 40 mV/s; initial potential: -0.50 V; switching potential: +0.35 V.

Figure 4a **Figure 4b** **Figure 4.**

Electropherogram of a mixture of 75 μM Ch, 75 μM ACh, 200 μM BuCh with detection at (a) HRP-Os/AChE-ChO modified Au microelectrode at +0.10 V and (b) PB/AChE-ChO modified Pt microelectrode at -0.10 V. Run buffer: 50 mM TES pH 8.0; separation voltage: +17 kV.

Figure 5. Electropherogram of a mixture containing 38 amol Ch, ACh, and BuCh detected by a HRP-Os/AChE-ChO Au microelectrode at +0.10 V. Run buffer: 50 mM TES pH 8.0; separation voltage: + 20 kV.

Figure 6.

Operational stability of HRP-Os/AChE-ChO and PB/AChE-ChO microelectrodes represented as normalized peak area ratios as a function of time. The peak ratio at time 0 is normalized to 1. Each point is the average response from three different electrodes to the injection of a 500 μ M solution of Ch, ACh and BuCh with equal molar amounts of each analyte. (◆)HRP-Os/AChE-ChO response to Ch; (■) HRP-Os/AChE-ChO response to ACh; (▲) PB/AChE-ChO response to Ch; (●) PB/AChE-ChO response to ACh.

Figure 7.

Operational stability of representative HRP-Os/AChE-ChO and PB/AChE-ChO microelectrodes in response to repetitive injections of a 500 μM solution of Ch, ACh and BuCh with equal molar amounts of each analyte. The ratio of the peak areas for Ch/BuCh and ACh/BuCh are shown as a function of number of runs performed. ($\blacklozenge$) HRP-Os/AChE-ChO response to Ch; ($\blacksquare$) HRP-Os/AChE-ChO response to ACh; ($\blacktriangle$) PB/AChE-ChO response to Ch; ($\bullet$) PB/AChE-ChO response to ACh.