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Disposable, enzymatically modified printed film carbon electrodes for use in the high-performance liquid chromatographic–electrochemical detection of glucose or hydrogen peroxide from immobilized enzyme reactors

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Abstract

Disposable screen-printed, film carbon electrodes (PFCE) were modified with cast-coated Osmium–polyvinylpyrridine-wired horse radish peroxidase gel polymer (Os-gel-HRP) to enable the detection of the reduction at 0 mV of hydrogen peroxide (H_2O_2) derived from a post-column immobilized enzyme reactor (IMER) containing acetylcholinesterase and choline oxidase. In another series of experiments PFCE were initially modified with cast-coated Os-gel-HRP and then treated with glucose oxidase in bovine serum albumin (BSA) and cross-linked with glutaraldehyde to form a bi-layer glucose–Os-gel-HRP PFCE. This bi-layer glucose–Os-gel-HRP PFCE generated a reduction current at 0 mV to H_2O_2 derived from the reaction of glucose oxidase and glucose in solution. These enzyme-modified PFCE were housed in a radial flow cell and coupled with cation-exchange liquid chromatographic methods to temporally separate substrates in solution for the determination of acetylcholine (ACh) and choline (Ch) in the first experimental series, or glucose in the second experimental series. These two disposable enzyme-modified PFCE exhibited linear current vs. substrate relations, were durable, being usable for approximately 40 determinations, and were sufficiently sensitive to be employed in biological sampling. Both assays utilized the same HPLC equipment. The limit of detection for ACh was 16 fmol/10 μl and that for glucose was 12 μmol/7.5 μl. ACh and Ch were measured from a microdialysate from the frontal cortex of a rat. Glucose in human urine was determined using the bi-layer glucose oxidase–Os-gel-HRP PFCE.

Keywords: Film electrodes; Glucose; Hydrogen peroxide

1. Introduction

At present, one of the most common methods for amperometric HPLC–ED assays of glucose, lactate, pyruvate and ACh/Ch from microdialysates is based on the temporal separation of substrates by chromatography coupled with the post-column, two-part process of the generation of H_2O_2, produced by the reaction of oxidase enzymes immobilized in an immobilized enzyme reactor (IMER) with their substrate in solution, and the subsequent detection of the generated H_2O_2 at the downstream electrochemical detector [1,2]. These analytical systems are very sensitive and well suited to the processing of large numbers of samples, however the drawbacks are that IMERs are expensive and prone to bacterial contami-

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nation whereupon bacterial catalase degrades H_2O_2, while the amperometric detection of the oxidation of H_2O_2 using platinum electrodes is subject to faradic interference. The recent development of hydrogels of peroxidases and redox polymers for use on glassy carbon electrodes for the detection of H_2O_2 at 0 mV has provided a means of significantly reducing the problem of faradic interference at platinum electrodes and also increased sensitivity [2,3].

Plastic and silicon film carbon electrodes are becoming increasingly employed in analytical procedures such as biosensing [4,5] and HPLC [6]. The majority of PFCE are produced by screen-printing techniques with pattern resolutions of 50 μm, although extremely high precision interdigitated silicon electrodes with pattern resolution of 2 μm are produced photolithographically [7]. These two methods, have the advantage over conventional glassy carbon electrode construction in that complex electrode geometries can be computer generated and the computer-controlled printing technique ensures accurate reproduction while production costs, once the template has been fabricated, are greatly reduced.

The conductance of the PFCE is derived from the use of a printing material or ink that incorporates particles capable of transferring electrons in an adhesive matrix. The content, size and species of the conductive particles, as well as other properties of the adhesive matrix, such as inclusion of enzymes [4,5], can be varied to optimize performance of the film electrodes for a range of applications.

We present data from two varieties of enzyme-modified screen-printed PFCE for use in flow cells coupled to HPLC–ED. In one experimental series a PFCE was modified by coating with Os-gel-HRP for the detection of H_2O_2 derived from an ACh/Ch IMER [2]. In a second experimental series, Os-gel-HRP on the PFCE was then cross-linked with glucose oxidase to form bi-layer glucose–Os-gel-HRP PFCE for use in HPLC–ED detection of glucose without the need for an IMER.

2. Experimental

2.1. Chemicals

All chemicals used were reagent grade and purchased from Takara (Osaka, Japan). Ascorbic acid, bovine serum albumin (BSA), butanone, glucose, glutaraldehyde and hydrogen peroxide (30%) were purchased from Takara. Acetylcholine chloride, choline chloride, L-glucose oxidase (from *Aspergillus niger* type VII) was purchased from Sigma (St. Louis, MO, USA). Osmium–polyvinylpyrridine-wired horse radish peroxidase gel polymer and ACh esterase–Ch oxidase IMER were purchased from BAS (West Lafayette, IN, USA). Ringers solution was prepared as follows: [NaCl]=150 m*M*; [KCl]=3 m*M*; [$MgCl_2$]=0.8 m*M*; [$CACl_2$]=1.2 m*M*.

2.2. PFCE

Disk PFCE (3 mm diameter; area=28.2 mm^2) were prepared by screen-printing carbon-based PVC glue on to the surface of PVC film. Once printed, the PFCE was heat cured and a removable plastic membrane was statically attached to protect the surface until the PFCE was to be cleaned and enzymatically modified. Conventional 3-mm diameter glassy carbon electrodes were purchased from BAS. Prior to testing, PFCE and glassy carbon electrodes were polished in a sonicator with a solution of 0.05-μm aluminum particles purchased from Buehler (Lake Bluff, MN, USA).

3. Results and discussion

3.1. Os-gel-HRP PFCE and glassy carbon electrodes

Os-gel-HRP (0.6 μl) was cast coated onto the surface of the 3-mm disk PFCE and glassy carbon electrodes. These electrodes were dried for at least 15 h before testing. In agreement with previous reports for glassy carbon electrodes modified with Os-gel-HRP [2], Os-gel-HRP PFCE quickly attained stabilization of background current (less than 1 h). Soaking the electrode in buffer at 4°C overnight reduced this time to about 30 min.

In preliminary experiments utilizing constant flow conditions, the reductive current generated at a 3-mm Osm-gel-HRP PFCE poised at 0 mV (vs. Ag/AgCl) in a flow cell with a 50-μm gasket to 2.5 μ*M* H_2O_2 in 0.15 *M* phosphate buffer–Ringers solution increased linearly with perfusion speeds ranging from 5 to 25 μl/min, thereafter current increase tended

towards saturation as speed increased above 50 μl/min. In constant-flow experiments the relation between reduction current vs. buffer–Ringers–H_2O_2 concentration was linear from 0.1 to 100 μ*M* H_2O_2 when a perfusion speed of 20 μl/min was employed (data not presented).

3.1.1. Acetylcholine and choline determination by HPLC–ED using Os-gel-HRP film or glassy carbon electrodes poised at 0 mV

Using standard HPLC–ED procedures [2] at room temperature, a standard solution containing ACh, Ch and ethylhomocholine (EHCh) (40, 100, 200, 1000 pmol/10 μl) was measured at 0 mV (vs. $Ag/AgCl_2$) with either Os-gel-HRP-coated PFCE or Os-gel-HRP-coated glassy carbon electrodes. Flow cell gasket size was 50 μm. The mobile phase (75 m*M* Na_2HPO_4–NaH_2PO_4, 0.5 m*M* EDTA–2Na, pH 8.0) was degassed (BAS CD-32 degasser) and pumped using a micro-LC pump (BAS LC-100) at 100 μl/min onto a polymeric cation-exchange microbore column (530×1 mm, BAS Sepstik unijet). ACh, EHCh and Ch were oxidized to H_2O_2 by a IMER (5 cm) containing ACh esterase and Ch oxidase [1,2]. For the analysis of dialysate samples the mobile phase was 50 m*M* Na_2HPO_4–NaH_2PO_4, 0.5 m*M* EDTA–2Na, (pH 8.4). Decreasing the $NaPO_4$ concentration increased the retention of ACh and Ch on the column thereby improving separation.

The advantages of Os-gel-HRP -coated glassy carbon electrodes in the experimental determination of H_2O_2 from biological samples is well established [1,2]. In the current experiments, ACh, EHCh and Ch were effectively determined using both the Os-gel-HRP-coated glassy carbon electrode or PFCE. Representative chromatographs for the detection of 40 fmol/10 μl of standard containing EHCh and ACh are presented in Fig. 1. The retention times and eluted peaks for analytes were very similar for both electrodes, however the magnitude of the current generated by Os-gel-HRP -coated PFCE was only 60% (n=5 determinations at 40, 100, 200 fmol/10 μl) of the current generated for equimolar solutions at the glassy carbon electrode. Although not optimized for maximum sensitivity the detection limit of this HPLC system was 10 and 16 fmol/10 μl for the glassy carbon and PFCE, respectively.

In bio-research laboratories platinum electrodes are still commonly employed in the amperometric

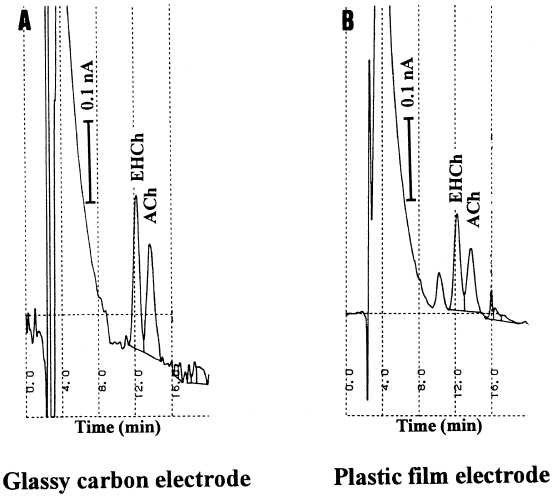

Fig. 1. HPLC–ED separations of 10 μl of a standard of 40 fmol ethylhomocholine (EHCh) and acetylcholine (ACh) with (A) Os-gel-HRP glassy carbon (3 mm diameter) or (B) Os-gel-HRP PFCE (3 mm diameter) as the cathode (for chromatographic procedures refer to text).

determination of H_2O_2. Fig. 2 shows the chromatographs of the same microdialysis perfusate (CMA 11 probe with a 4-mm membrane perfused at 1 μl/min with Ringers solution) from the frontal cortex of a rat

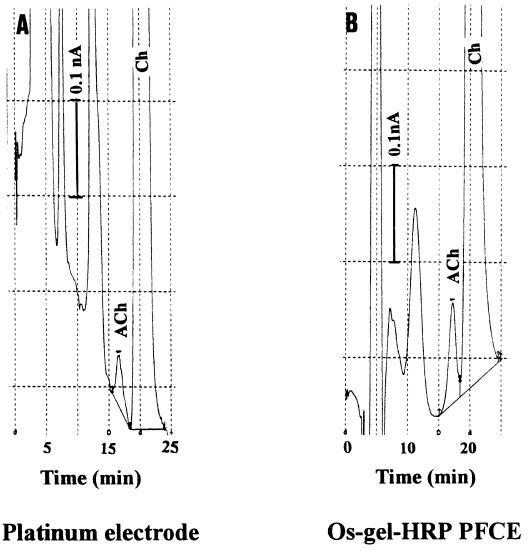

Fig. 2. HPLC–ED separations of 10 μl of the same dialysate sample from the prefrontal cortex of a rat using identical chromatographic procedures, but the detector was (A) a 3-mm platinum electrode poised at 450 mV or (B) a 3-mm Os-gel-HRP PFCE (for chromatographic procedures refer to text).

that was analysed on either a platinum electrode (450 mV vs. Ag/AgCl) or on an Os-gel-HRP PFCE (0 mV vs. Ag/AgCl). Ch was quantifiable using both electrode types. ACh was quantifiable using the Os-gel-HRP PFCE (104 fmol/10 μl as determined by comparison with a standard solution) but was smaller and less symmetrical using the platinum electrode. For these determinations flow cell gasket thickness for both electrodes was 25 μ*M*.

3.1.2. Stability

Fig. 3 shows the reduction current response (peak height) of 3-mm Os-gel-HRP-coated PFCE to 40 consecutive standards of ACh (1 pmol/10 μl injection) maintained at 4°C injected using a CMA 200 autosampler. The current steadily decreased with each injection having been reduced by 30% after 40 chromatographic runs, each of 25 min length. Samples 1–7 were standards of other concentrations. It is probable that the occasional irregularities in reduction current between samples result from small variations in the amount of substrate autoinjected.

3.2. Bi-layer glucose oxidase-Os-gel-HRP PFCE

Bi-layer glucose oxidase–Os-gel-HRP PFCE were fabricated using a variation on published techniques [8]. The Os-gel-HRP-coated PFCE was cast-coated with 0.7 μl of glucose oxidase (1.8% by weight) in 1% BSA in 0.15 *M* PBS (pH 5.7) and 1% glutaraldehyde in 0.15 *M* PBS (pH 5.7), and then dried at room temperature (24°C) at high humidity for 2 h. The electrode was stored at 4°C until used.

3.2.1. Amperometric determination of glucose using bi-layer glucose oxidase-Os-gel-HRP PFCE with HPLC separation

Flow cell gasket size was 50 μm. The mobile phase (25 m*M* Na_2HPO_4–NaH_2PO_4, pH 5.7) was degassed (BAS CD-32 degasser) and pumped using a micro-LC pump (BAS LC-100) at 60 μl/min onto a polymeric cation-exchange microbore column (530× 1 mm, BAS Sepstik unijet). Samples and standards (7.5 μl) were maintained at 4°C and injected using a CMA 200 autosampler. The bi-layer glucose oxidase–Os-gel-HRP PFCE quickly stabilized background current and was ready to use less than 45 min after inserting into the flow cell. Using the above chromatographic configuration, glucose eluted from the column about 1.5 min after the front. Fig. 4 shows four overlapping chromatographs of (a) 200 μ*M* glucose (upper trace), (b) urine of a person with a reduced urinary glucose excretion threshold (sample diluted with Ringers by 50%), (c) Ringers

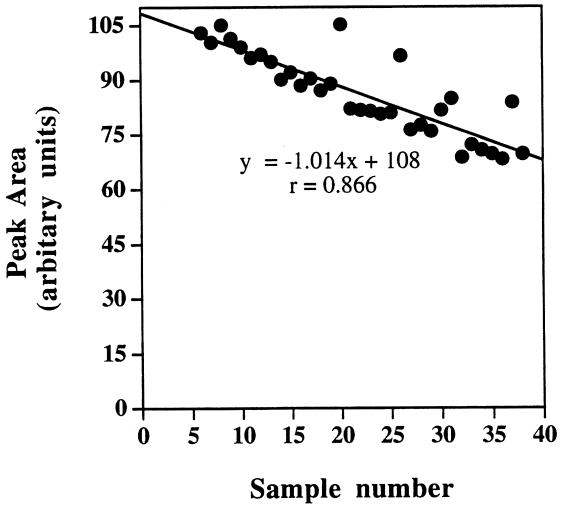

Fig. 3. Current response (peak area in arbitrary units) to 40 injections of standards of ACh standards solutions (1000 fmol/10 μl). Samples 1–7 were other concentrations of ACh. The equation of least-squares best fit is written.

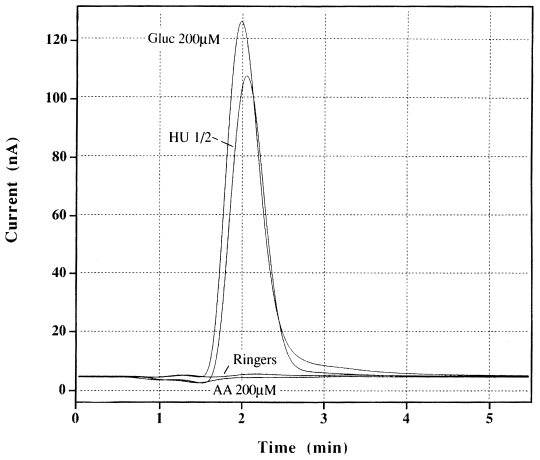

Fig. 4. Overlapping chromatographs of 7.5-μl injections of 200 μ*M* glucose in Ringers (upper trace), human urine diluted 50% with Ringers solution (HU 1/2), Ringers solution and 200 μ*M* ascorbic acid in Ringers (lower trace) (for chromatographic details refer to text).

solution and (d) 200 μ*M* ascorbic acid in Ringers solution.

3.2.2. Stability

Fig. 5 shows the maximum reductive current to standards (12.5– 250 μ*M*), the progressive decrease of current in response to 40 injections of standards of glucose (100 μ*M*/7.5 μl injection) and a limited repetition of the standard curve (50–250 μ*M*). The bi-layer glucose Os-gel-HRP PFCE exhibited a linear relation between reduction current and glucose concentration over the range of 12.5–1000 μ*M* (seven-point standard curve, $y=0.461x+2.2$, $R=0.999$). The reduction current to 100 μ*M* glucose (the approximate concentration obtained from the smallest commercially available microdialysis probes under standard experimental conditions) [8] was large (approximately 50 nA) and decreased with each successive injection. After 40 injections the current had decreased by 37%, however the current remained well within the workable range and a linear relation between current and substrate concentration was retained (see insert, Fig. 5). Faradic interferents, such as ascorbic acid, were eluted in the front and the magnitude of this interference was minimized by maintaining the electrode potential at 0 mV (vs. Ag/AgCl) and utilizing a low-pH buffer which also maximized glucose oxidase activity. In continuous-flow experiments, the bi-layer glucose oxidase–Os-gel-HRP PFCE exhibited similar stability and reactivity in other buffers, such as lithium acetate buffers or buffers containing 20–150 m*M* Na_2HPO_4–NaH_2PO_4, and 10% acetonitrile. In chromatographic separations, the lithium acetate buffer could be expected to enhance the retention of glucose on the cation-exchange column. The bi-layer glucose oxidase–Os-gel-HRP PFCE displayed modest reduction in activity after dry storage at 4°C for 2 weeks.

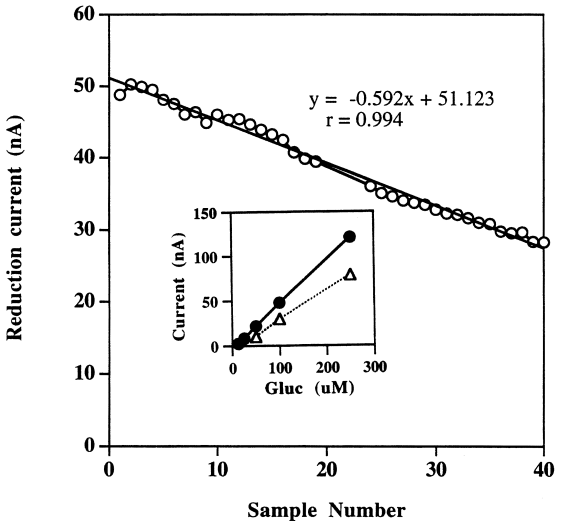

Fig. 5. Peak current (nA) recorded in response to glucose standard solutions (12.5–250 μ*M*) (insert, solid circles), the progressive decrease of current in response to 40 injections of standards of glucose (100 μ*M*) (main graph, open circles) and a subsequent repetition of the standard curve after (50–250 μ*M*) (insert, open triangles). Samples 19–23 were other concentrations of glucose and Ringers.

4. Conclusions

Increasingly, bio-medical knowledge is benefiting from greater understanding of the in vivo interactions of chemically unrelated neuroactive substances. However, analysis of each analyte often requires specific analytical hardware in addition to a high degree of user technical proficiency. In this research environment inexpensive, easy to operate, enzyme-modified electrodes that could be incorporated into existing technologies could prove useful.

Disposable PFCE, modified with Os-gel-HRP for the detection of H_2O_2 from IMER and bi-layer glucose oxidase–Os-gel-HRP PFCE for the measurement of glucose without the need for an IMER, when incorporated into existing HPLC-ED techniques, provide a useful simplification of standard chromatographic assays. These two enzyme-modified PFCE, respectively, enable the measurement of ACh, Ch and glucose using exactly the same HPLC equipment. As methods for immobilization of enzymes improve, so that specificity is not compromised by cross-linking [9], an increasing variety and complexity of disposable bi-layer PFCE will become available as research tools.

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