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'Wiring' of glucose oxidase and lactate oxidase within a hydrogel made with poly(vinyl pyridine) complexed with [Os(4,4′-dimethoxy-2,2′-bipyridine)$_2$Cl]$^{+/2+}$

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Glucose and lactate electrodes based on hydrogels made by crosslinking glucose oxidase and the redox polymer formed upon complexing poly(vinyl pyridine) (PVP) with [Os(dmo-bpy)$_2$Cl]$^{+/2+}$ (dmo-bpy = 4,4′-dimethoxy-2,2′-bipyridine) on vitreous carbon electrode surfaces have been investigated. The redox potential of the hydrogels was +35 mV *vs.* SCE and their glucose electrooxidation current reached a plateau at +150 mV *vs.* SCE. Urate and acetaminophen were not electrooxidized at this potential at rates that would interfere with the glucose and lactate assays. At a glucose concentration of 1 mM, the addition of 0.1 mM ascorbate increased the current by 17%. At 5 mM glucose, switching the atmosphere from argon to oxygen reduced the currents by 11%.

In vivo sensing of glucose and lactate is relevant to medicine. In the case of glucose, the management of diabetes mellitus would be improved by employment of a continuous *in vivo* glucose sensor. Lactate is a metabolite of anaerobic cellular glucose metabolism. A continuous *in vivo* lactate sensor would be of relevance to treatment of respiratory insufficiency, shock and heart failure.

Glucose and lactate electrodes that do not require oxygen for their operation and have no leachable components have been made by electrically 'wiring' glucose oxidase (GOx) and lactate oxidase (LOx). In these, a crosslinked redox polyelectrolyte network, to which the enzyme was covalently bound, formed an electron conducting hydrogel on the surface of the electrode. Because the hydrogel conducted electrons through electron transfer between the [Os(bpy)$_2$Cl]$^{+/2+}$ redox centres, bound to either PVP or poly(vinyl imidazole) (PVI), and because it was permeable to water-soluble substrates and products, the current densities and sensitivities of the resulting electrodes were high.[1–4]

Like the enzyme electrodes based on diffusional mediators, these wired enzyme sensors functioned also under anaerobic conditions. However, unlike enzyme electrodes based on diffusional mediators, they could be used in flow systems; because of the absence of leachable mediator, the redox couple being covalently bound to the hydrogel.

The specificity of amperometric biosensors depends on the potential at which they are poised. At enzyme electrodes on which substrates are electrooxidized the rate of electrooxidation of interferents increases with increasing oxidizing potentials. In enzyme electrodes the merit of using diffusional redox mediators of relatively reducing redox potentials, having at pH 7 standard potentials that are closer to that of the enzyme, is well recognized.[5] The electrooxidizable interferent-caused currents are reduced upon shifting the redox potentials of the hydrogels to more reducing potentials so as to allow the poising of the electrodes in current plateaus at more negative potentials. Earlier the potentials were sequentially down-shifted by complexing osmium bis-2,2′-bipyridine chloride, [Os(bpy)$_2$Cl]$^{+/2+}$, to imidazole nitrogens of PVI rather than to pyridine nitrogens in PVP,[4,6,7] then by using 4,4′-dimethyl substituted 2,2′-bipyridines, with which a polymer denoted as PVI-dme-dpy-Os was made,[8] and then subsequently by using 4,4′-dimethoxy substituted 2,2′-bipyridines, with which a polymer denoted as PVI-dmo-bpy-Os was made. The glucose electrode made with the hydrogel based on the latter polymer proved to be insensitive to urate and acetaminophen. However, ascorbate electrooxidation still contributed to the current, and the electrodes based on this polymer had operational half lives of only 2–3 h and were also sensitive to oxygen.[9] Here it is shown that (*i*) the osmium bis-4,4′-dimethoxy substituted 2,2′-bipyridine chloride complex of a partially methylated PVP (denoted PVP-dmo-bpy-Os) effectively wires both glucose oxidase and lactate oxidase; (*ii*) the redox potential of this polymer is +35 mV *vs.* SCE; (*iii*) electrodes made with it, when poised at +200 mV *vs.* SCE, are insensitive to urate and acetaminophen and are also less sensitive to ascorbate interference and oxygen competition than wired enzyme electrodes made with the previously reported polymers; and (*iv*) the operational half-life of the enzyme electrodes, rotating at 1000 rpm, is 16 h at 21 °C.

Experimental

Chemicals

Glucose oxidase (GOx) (Sigma, EC 1.1.3.4) from *Aspergillus niger* [type X-S, 198 units (mg of solid)$^{-1}$, 75% protein], lactate oxidase (LOx) (Genzyme, EC 1.1.3.2) from *Pediococcus sp.* [28.0 units (mg of solid)$^{-1}$, 45% protein], poly(ethylene glycol) diglycidyl ether (PEGDGE) (Polysciences Inc.), ammonium hexachloroosmate(IV) (Johnson Matthey) and poly(4-vinyl pyridine) (M_r = 50000) (Reilly) were used as received. Glucose solutions were prepared by diluting a stock 2 M solution in phosphate buffer. All other chemicals were from Aldrich and were used without further purification.

Synthesis of PVP-dmo-bpy-Os

4,4′-dimethoxy-2,2′-bipyridine (dmo-bpy) was prepared as described elsewhere[7,10] except for modification of the nitration step. 4,4′-dinitro-2,2′-bipyridine-*N*,*N*′-dioxide was made by dissolving 10 g 2,2′-bipyridine-*N*,*N*′-dioxide in 40 ml fuming (1.9 g cm^{-3}) sulfuric acid, cooled in an ice bath. 30 ml fuming nitric acid (1.48 g cm^{-3}) was added dropwise and the mixture was kept at 100 °C for 4 h. After cooling to room temperature, the mixture was poured over a minimum amount of ice, and the product was collected by filtering through a glass frit. The yield was *ca.* 55%. The nitro groups

were substituted by methoxy groups and the *N*-oxide was deoxygenated according to reported procedures.[9,10]

The preparation of the $Os(dmo\text{-}bpy)_2Cl_2$ was carried out under argon. 200 mg of dmo-bpy and 202 mg $(NH_4)_2OsCl_6$ were mixed with 7 ml ethylene glycol, the solution was degassed by bubbling argon and heated for 1 h at reflux (*ca.* 200 °C). The mixture was then cooled to room temperature and an aqueous solution of sodium dithionite (10 ml, 1 M) was added. The sodium dithionite reduced the complex, which precipitated. The mixture was stirred in an ice bath for 30 min prior to filtering on a fritted glass filter. The yield was 90%.

The preparation of the enzyme wiring polymer was carried out under argon. 300 mg of $Os(dmo\text{-}bpy)_2Cl_2$ and 180 mg of poly(4-vinyl pyridine) were added (1 : 4 molar ratio) to 20 ml of ethylene glycol. The reaction mixture was heated at 160 °C for 3 h in a 100 ml round-bottomed flask fitted with a reflux condenser. The reaction mixture was then precipitated dropwise into 1000 ml of ethyl acetate, and the resulting solid was redissolved into 20 ml water. The solution was then passed through a Sephadex G-25 size exclusion column. Two distinct bands formed on the column. The first contained the desired high-molecular-weight redox polymer. The subsequent band contained by-products and unreacted starting materials. 5 g of Bio-Rad AG 1-X8 ion-exchange beads were added to the polymer solution, and the mixture was stirred for 16 h at room temperature. The beads were removed by filtering. The polymer solution was collected and dialysed in a 3500 M_r cutoff bag against water for two days in order to remove all the salts. The dialysed solution was evaporated and the product, PVP_{12}-Os-dmo-bpy, was collected. The yield was 75%. Calc. for PVP_{12}-Os-dmo-bpy · $7H_2O$ $C_{108}H_{122}Cl_2N_{16}O_{11}Os$: C, 62.34; H, 5.87; Cl, 3.42; N, 10.77; Os, 9.14. Found: C, 64.14; H, 6.29; Cl, 3.46; N, 9.31; Os, 8.76.

30% of the unreacted pyridine rings of the resulting polymer were methylated according to previously published procedures.[11,12] 250 mg of the polymer were redissolved into 15 ml of ethylene glycol in a 100 ml round-bottomed flask fitted with a reflux condenser. 60 mg of CH_3I and 15 ml DMF were added to the mixture which was stirred for 24 h at 75 °C. The polymer was precipitated, separated, ion-exchanged, dialysed and collected as described above for the non-methylated polymer.

PVI-dmo-bpy-Os and PVI-dme-bpy-Os were prepared according to previously published procedures.[8,9]

Electrodes

3 mm diameter vitreous carbon electrodes were used. These were polished and cleaned using 3 grades of alumina slurry (5, 1, 0.3 μm) with sonication and rinsing between grades. They were tested in phosphate buffer by scanning between the potentials of interest (from −0.4 to 0.4 V *vs.* SCE) to assure that their electrochemistry was featureless. The wired enzyme electrodes were prepared, unless otherwise noted, by placing droplets of 4 μl of the PVP-dmo-bpy-Os (10 mg ml^{-1}), 2 μl of enzyme (9 mg ml^{-1}) and 1 μl of the crosslinker PEGDGE (5.8 mg ml^{-1}) on the glassy carbon surface and mixing with the tip of a syringe. The electrodes were then placed in an evacuated desiccator for 16 h prior to use. Their dark-purple films appeared to be uniformly spread.

Measurements

The measurements, unless otherwise noted, were carried out under argon, using a Princeton Applied Research model 273 potentiostat/galvanostat in a three-electrode cell. Rotating-disc experiments were performed using a Pine RDE4 potentiostat, with an MSRX speed controller, an *X*–*Y*–*Y*′ plotter and a VWR 1165 refrigerated constant temperature circulator. All measurements, except those in which the pH was varied, were performed using a 20 mM phosphate buffer (pH 7.3) containing 0.1 M NaCl. In experiments where the pH was varied, 2 M solutions of HCl or NaOH were added to the phosphate buffer solution. The rotating disc experiments were run at 21.3 °C in 50 ml of phosphate buffer in a cell having a rotating (1000 rpm) glassy carbon working electrode, a saturated calomel reference electrode (SCE) and a platinum counter-electrode, isolated from the bulk solution by a Vycor frit. The flow-cell measurements were performed in air using a Cole-Parmer Ismatec SA vario-pump system, set to 1.2 ml min^{-1}. The cell had a void volume of 1 ml. The flow cell consisted of two glassy carbon working electrodes, a platinum counter-electrode and an SCE reference electrode connected to the RDE4 potentiostat and *X*–*Y*–*Y*′ plotter.

Results and Discussion

Cyclic voltammetry of PVP-dmo-bpy-Os modified vitreous carbon electrodes

Fig. 1 shows the cyclic voltammogram of the PVP-dmo-bpy-Os modified vitreous carbon electrode at a 5 mV s^{-1} scan rate. The oxidation and reduction peaks were separated by 42 mV. The redox potential of the polymer, when crosslinked with PEGDGE-400, was +44 mV *vs.* SCE. The amount of electroactive osmium present in the hydrogel was determined by integration of the oxidation peak of a 5 mV s^{-1} cyclic voltammogram and was found to be 65 ± 6 μC for all PVP-dmo-bpy-Os electrodes except those with loadings of less than 20 μg. The crosslinked hydrogels adhered well to the electrodes and retained *ca.* 90% of their electroactive osmium when soaked in a stirred solution of phosphate buffer for 48 h.

Potential dependence of steady-state glucose and lactate response of crosslinked PVP-dmo-bpy-Os based hydrogels

The potential dependence of the electrocatalytic oxidation current is shown for a glucose electrode in Fig. 2. Lactate electrodes exhibited the same potential dependence. To assure independence of the electrooxidation currents of minor perturbations in the applied potential, the electrodes were poised at +200 mV *vs.* SCE, well within the current plateau.

Dependence of steady-state glucose and lactate response on amount of immobilized enzyme in the hydrogel

The steady-state electrooxidation current for glucose and lactate was measured at 1000 rpm as a function of the weight fraction of enzyme in the films at 64 mM glucose and 2 mM lactate, respectively. These concentrations greatly exceeded the respective apparent Michaelis constants ($K_{m,app}$) of the electrodes. In the glucose electrodes, the hydrogels consisted of a

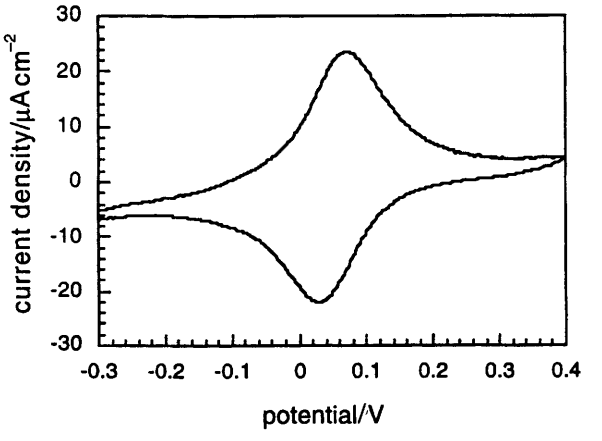

Fig. 1 Cyclic voltammogram of a typical PVP-dmo-bpy-Os glucose electrode with 40 μg PVP-dmo-bpy-Os, 18 μg GOx, 5.8 μg PEG-400; scan rate = 5 mV s^{-1}; under air; T = 21.3 °C

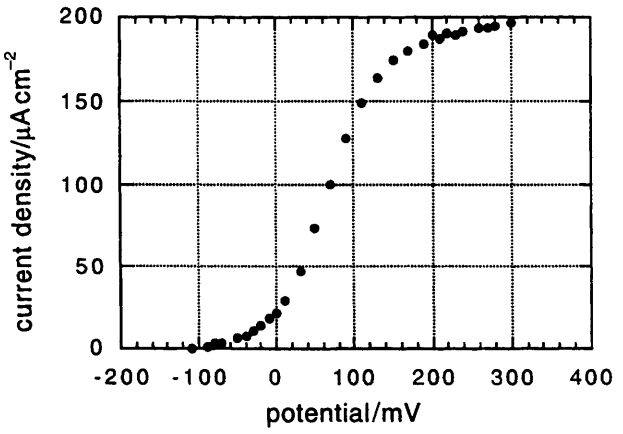

Fig. 2 Potential dependence of the current density for a typical PVP-dmo-bpy-Os glucose electrode with 20 μg PVP-dmo-bpy-Os, 9 μg GOx, 2.9 μg PEG-400; 20 mM glucose; under argon; 1000 rpm; $T = 21.3$ °C

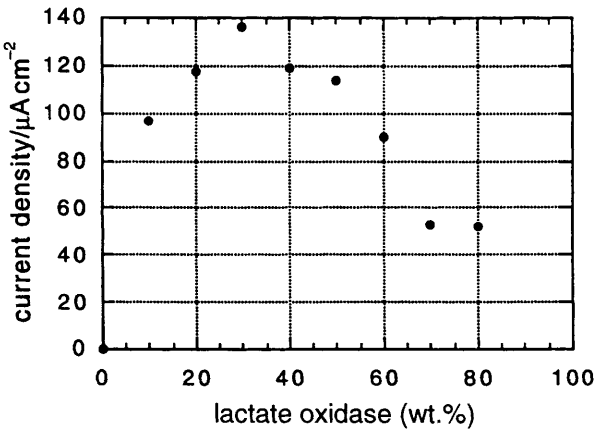

Fig. 4 Dependence of lactate electrooxidation current densities on LOx (wt.%) for electrodes with 5 μg PVP-dmo-bpy-Os, 0.5–12.5 μg LOx, 10 wt.% PEG-400; 6 mM lactate; under argon; 1000 rpm; $T = 21.3$ °C

fixed amount of PVP-dmo (40 μg), 4–100 μg of GOx and 10% PEG-400 (by weight). The currents were highest in electrodes containing between 10 and 17.2 μg of GOx, corresponding to 20–30 wt.% of enzyme (Fig. 3). In this range of enzyme weight fraction, the current density for electrocatalytic oxidation of 64 mM glucose was 260 μA cm^{-2}. The apparent Michaelis constant, K_m, was determined through a Lineweaver–Burke plot. For 600 μg cm^{-2} loading with 30 wt.% GOx, K_m was 1.85 mM glucose. In a similar study for lactate, the hydrogels consisted of a fixed amount of PVP-dmo (5 μg), 0.5–12.5 μg of LOx and 10% PEG-400 (by weight). The lactate currents were highest in electrodes containing 4.3 μg of LOx, corresponding to 30 wt.% (Fig. 4). With this amount of enzyme, the current density at 2 mM lactate was 138 μA cm^{-2}. The apparent K_m for the electrode with 75 μg cm^{-2} loading with 30 wt.% LOx with 0.32 mM lactate.

Dependence of the steady-state glucose and lactate response on film thickness

In the glucose assays, the electrodes had a fixed GOx to PVP-dmo weight ratio of 7 to 3 with a weight fraction of PEG of 10% of the combined PVP-dmo and GOx weights. As shown in Fig. 5, the glucose response at PVP-dmo loadings greater than 450 μg cm^{-2} (*i.e.* 30 μg), was independent of PVP-dmo loading. In this range of loadings, the current density was 290 μA cm^{-2}. Films thicker than 600 μg cm^{-2} were difficult to reproduce, their current densities varying by ±10%. Nevertheless, when the current densities were normalized by their coulometrically determined electroactive osmium content, the scatter was reduced to ±1.5%.

Similar lactate electrodes were prepared also at a LOx to PVP-dmo weight ratio of 7 to 3 with 10% by weight of PEG. As shown in Fig. 6, the lactate response at PVP-dmo loadings of greater than 150 μg cm^{-2} (*i.e.* 30 μg) was independent of

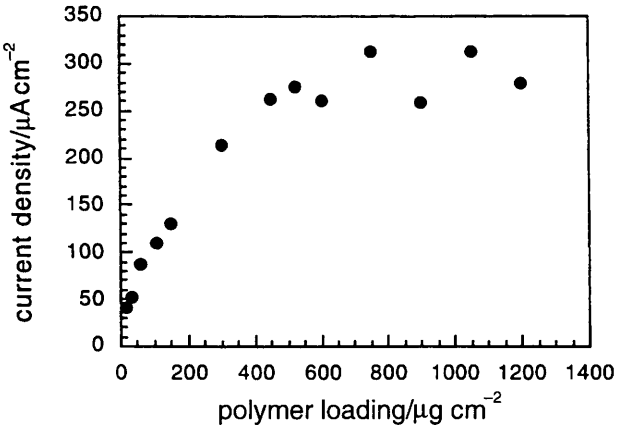

Fig. 5 Dependence of glucose electrooxidation current densities on PVP-dmo-bpy-Os loading for electrodes with 30 wt.% GOx; 20 mM glucose; under argon; 1000 rpm; $T = 21.3$ °C

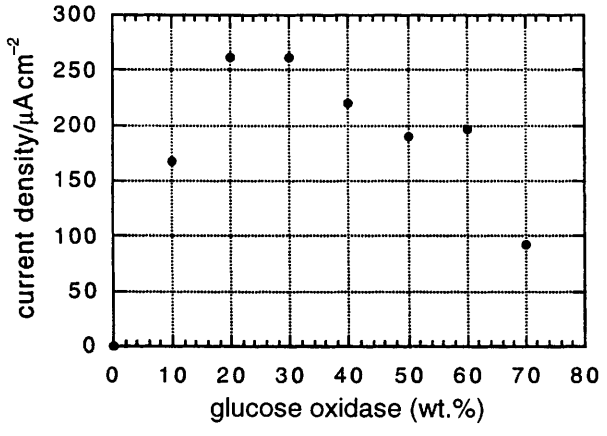

Fig. 3 Dependence of glucose electrooxidation current densities on GOx (wt.%) for electrodes with 40 μg PVP-dmo-bpy-Os, 4–100 μg GOx, 10 wt.% PEG-400; 20 mM glucose; under argon; 1000 rpm; $T = 21.3$ °C

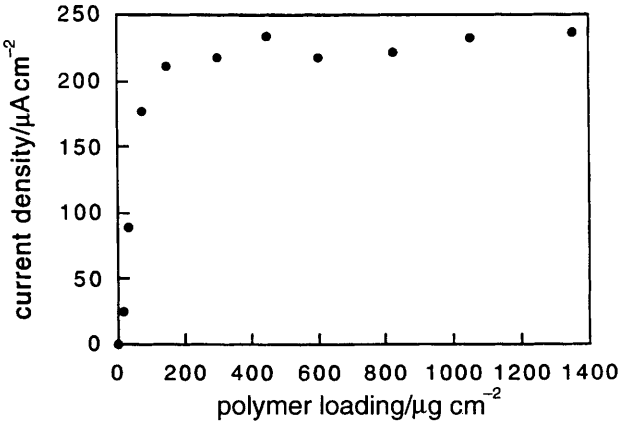

Fig. 6 Dependence of the lactate electrooxidation current densities on PVP-dmo-bpy-Os loading for electrodes with 30 wt.% LOx; 6 mM lactate; under argon; 1000 rpm; $T = 21.3$ °C

the PVP-dmo loading. In this range, the current density was 230 μA cm^{-2}.

Dependence of steady-state glucose and lactate response on pH

The dependence of current density on pH is shown, for glucose, in Fig. 7 and, for lactate, in Fig. 8. As in the case of other osmium based polymers,[7,9,13,14] the catalytic currents for both the glucose and lactate sensors peaked and plateaued at pH 8.0–10.0 and were nearly independent of pH in this range. The electrodes were irreversibly deactivated at pH >10.

Effect of oxygen on the steady-state glucose and lactate response

The glucose concentration dependence of the electrocatalytic oxidation current density of a typical crosslinked glucose electrode (20 μg PVP-dmo, 9 μg GOx, 2.9 μg PEG-400) under O_2 and Ar at 1000 rpm is shown in Fig. 9. Fig. 10 shows the lactate concentration dependence of a lactate electrode (5 μg PVP-dmo, 2.25 μg LOx, 0.73 μg PEG-400) under the same conditions. As is evident, O_2 competes with the PVP-dmo-bpy-Os in the oxidation of the $FADH_2$ centres of the enzymes. The losses in current density (because of oxygen competition for electrons of the $FADH_2$ sites) are compared for three enzyme wiring redox hydrogels in Table 1. PVP-dmo-bpy-Os is a better wire than the other polymers, the loss

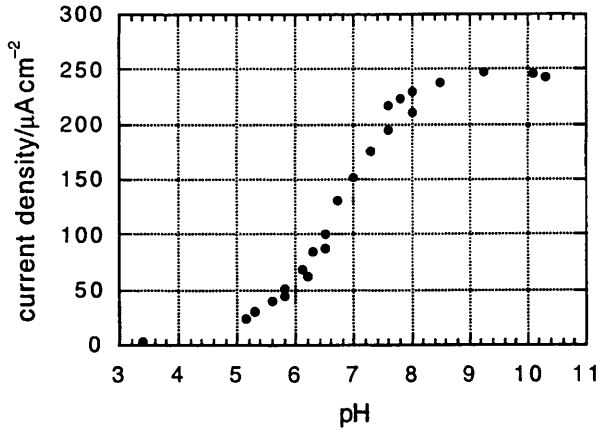

Fig. 7 pH dependence of the glucose electrooxidation current density in a typical PVP-dmo-bpy-Os based electrode with 40 μg PVP-dmo-bpy-Os, 18 μg GOx, 5.8 μg PEG-400; 20 mM glucose; under argon; 1000 rpm; T = 21.3 °C

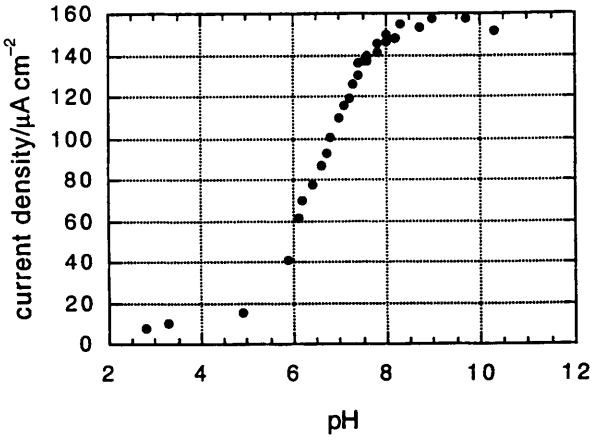

Fig. 8 pH dependence of the lactate electrooxidation current density in a typical PVP-dmo-bpy-Os based electrode with 5 μg PVP-dmo-bpy-Os, 2.25 μg LOx, 0.73 μg PEG-400; 6 mM lactate; under argon; 1000 rpm; T = 21.3 °C

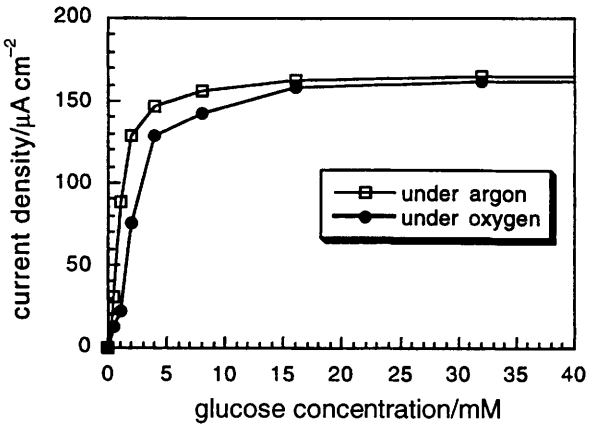

Fig. 9 Calibration curve for a typical PVP-dmo-bpy-Os glucose electrode with 20 μg PVP-dmo-bpy-Os, 9 μg GOx, 2.9 μg PEG-400; 20 mM glucose; 1000 rpm; T = 21.3 °C

in current density upon switching from an argon to an oxygen atmosphere being smaller in enzyme electrodes made with this polymer.

Electrodes based on PVP-dmo-bpy-Os differed remarkably from their PVI derived analogues in that O_2 was not electrocatalytically reduced on the PVP-derived electrodes. Fig. 11 shows the potential dependence of the O_2 electroreduction currents of glucose electrodes made with PVP-dmo-bpy-Os and PVI-dmo-bpy-Os in the absence of glucose. While O_2 is electroreduced at pH 7.4 on the PVI-dmo-bpy-Os electrodes at potentials negative of +20 mV *vs.* SCE, it is not electroreduced on the PVP-dmo-bpy-Os electrodes. The electroreduction of O_2 on PVI-dmo-bpy-Os electrodes is the subject of other work.[15]

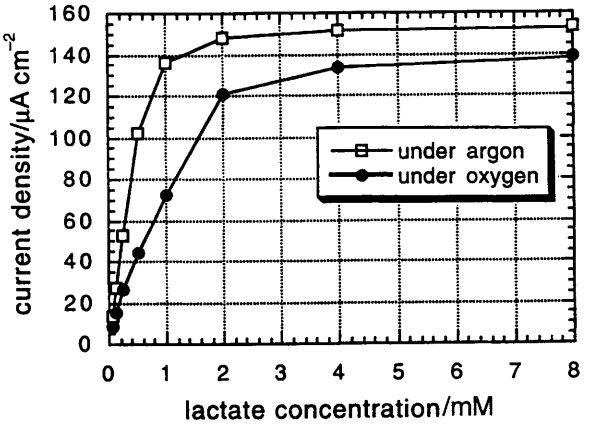

Fig. 10 Calibration curve for a typical PVP-dmo-bpy-Os lactate electrode with 5 μg PVP-dmo-bpy-Os, 2.25 μg LOx, 0.73 μg PEG-400; 6 mM lactate; 1000 rpm; T = 21.3 °C

Table 1 Loss of glucose electrooxidation current density for a typical glucose electrode (40 μg polymer, 18 μg GOx, 5.8 μg PEG-400) when switching from an argon atmosphere to an oxygen atmosphere at various glucose concentrations

	current decrease due to O_2 (%)		
polymer	2 mM glucose	5 mM glucose	32 mM glucose
PVP-dmo-bpy-Os	41	11	2
PVI-dme-bpy-Os	76	70	19
PVI-dmo-bpy-Os	47	26	16

1000 rpm; T = 21.3 °C.

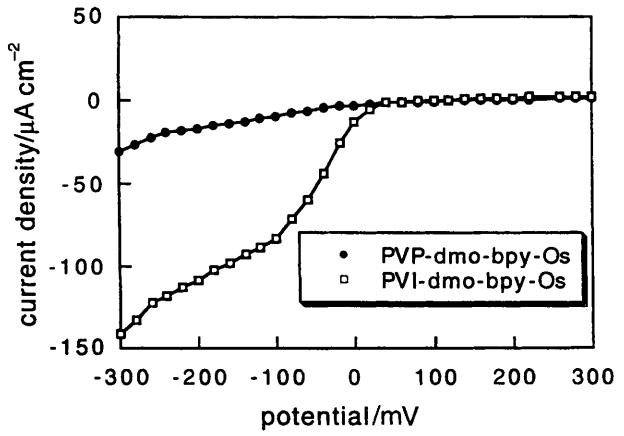

Fig. 11 Potential dependence of the oxygen electroreduction current density for typical glucose electrodes made with PVP-dmo-bpy-Os and PVI-dmo-bpy-Os (40 μg polymer, 18 μg GOx, 5.8 μg PEG-400); in the absence of glucose; 1000 rpm; T = 21.3 °C

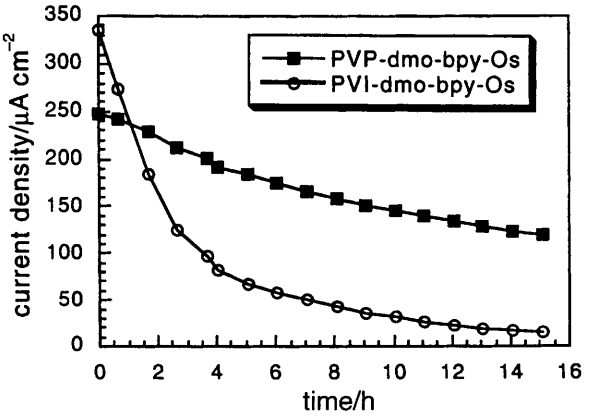

Fig. 12 Comparison of the operational stability of typical lactate electrodes made with PVP-dmo-bpy-Os and PVI-dmo-bpy-Os (40 μg polymer, 18 μg LOx, 5.8 μg PEG-400); 6 mM lactate; under argon; 1000 rpm; T = 21.3 °C

Selectivity

Glucose sensors are often insufficiently selective because their operation requires their poising at potentials where common interferents, such as urate, acetaminophen and ascorbate, are electrooxidized. The selectivity of glucose sensors can be improved by employing membranes that preferentially transport glucose. For example, Nafion[16,17] and cellulose acetate[18–20] have proven effective at reducing the error introduced by interferents. Lowering the operating potential reduces the rate of interferent electrooxidation.[8,9]

Comparison of the polymers, PVI-dme-bpy-Os ($E_{1/2}$ = +100 mV *vs.* SCE), PVI-dmo-bpy-Os ($E_{1/2}$ = −69 mV *vs.* SCE) and PVP-dmo-bpy-Os ($E_{1/2}$ = +35 mV *vs.* SCE), reveals that, at low glucose concentrations, the PVP-dmo-bpy-Os based glucose electrode is the most selective against ascorbate (Table 2). A 16.6% increase in current is produced by adding 0.1 mM ascorbate at 1 mM glucose for PVP-dmo-bpy-Os, while with PVI-dme-bpy-Os the current increases by 66.9% and with PVI-dmo-bpy-Os, by 97.2%. At higher concentrations of glucose, all three polymers behave similarly, with about a 14% increase in current from 0.1 mM ascorbate at 5 mM glucose. The rates of electrooxidation of urate and acetaminophen are negligibly slow for both PVP-dmo-bpy-Os and PVI-dmo-bpy-Os, whereas on PVI-dme-bpy-Os electrodes, both are electrooxidized.

Operational stability

The operational stabilities of a typical PVP-dmo-bpy-Os based lactate electrode (40 μg PVP-dmo, 18 μg LOx, 5.8 μg PEG-400) and a typical PVI-dmo-bpy-Os based lactate electrode (40 μg PVI-dmo, 18 μg LOx, 5.8 μg PEG-400) rotating at 1000 rpm are compared in Fig. 12. The half-life of the PVP-dmo-bpy-Os system is 16 h compared to 2 h for the PVI-dmo-bpy-Os system. For both systems this decay is accompanied by a proportional decrease in the amount of electroactive redox polymer measured through cyclic voltammetry, showing that, in the rapidly rotating electrodes, the main cause of the instability is the hydrodynamic shear-caused detachment of the redox hydrogel from the electrode surface.

Conclusion

Crosslinked, electron conducting hydrogels based on the polymer PVP-dmo-bpy-Os provide for improved amperometric sensors of glucose and lactate. PVP-dmo-bpy-Os based electrodes display optimal current densities at an enzyme weight fraction of 30% at loadings exceeding 350 μg cm^{-2} of PVP-dmo-bpy-Os with GOx and LOx. These PVP-dmo-bpy-Os based electrodes are more selective and stable than those based on other electron conducting hydrogels and are also less sensitive to oxygen competition, particularly at low glucose concentrations.

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Table 2 Comparison of the responses to common interferents of typical glucose electrodes based on different redox polymers (40 μg polymer, 18 μg GOx, 5.8 μg PEG-400)

	current increase due to 0.1 mM ascorbate (%)		current density/μA cm^{-2}		
polymer	1 mM glucose	5 mM glucose	0.1 mM ascorbate	0.5 mM urate	1 mM acetaminophen
PVP-dmo-bpy-Os	16.6	14.3	22.1	0	0
PVI-dme-bpy-Os	66.9	13.7	45	45	4.5
PVI-dmo-bpy-Os	97.2	14.6	51.8	0	0

Under argon; 1000 rpm; T = 21.3 °C.

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