A flexible lactate electrode was made of 400±100 μm diameter carbon fibers, epoxy embedded in a 0.3 mm diameter polyimide tubing. The electrode was modified by precipitating on it the relatively insoluble complex formed between 1100 kd partially N-ethylamine quaternized poly[(vinylpyridine)Os(bipyridine)2Cl] Cl (POs-EA) and lactate oxidase. The steady-state lactate electrooxidation current, at 2mM lactate concentration and at 22°C, was 400 nA. The 50±10 μA cm⁻² current density and the 20 mA cm⁻² M⁻¹ sensitivity decreased only by 5% upon increasing the partial pressure of oxygen from 0.0 to 0.2 atm. The electrode retains its sensitivity after dry storage at 4°C for 4 months in air but loses 37°C half of its sensitivity in 7 hours through polymer desorption when operated at 0.4 V (SCE). To eliminate interference by species that are electrooxidized at 0.4 V (SCE), the lactate sensing probe was (a) electrically insulated with an epoxy made of poly(vinylimidazole) cross-
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oligosaccharides; aldehydes.

linked with ethylene glycol diglycidyl ether, and (b) coated with an immobilized horseradish peroxidase (HRP)-glucose oxidase (GOX) film. The latter film was formed by co-immobilizing the two enzymes through periodate oxidation of their oligosaccharides to aldehydes and forming Schiff's-bases between the polyaldehydes and the enzymes' lysyl amines. In the presence of 1 mM glucose and in air, the interfering electrooxidation of 0.1 mM ascorbate was reduced by a factor of 20. This reduction results from formation of hydrogen peroxide in the glucose catalyzed reaction, and H$_2$O$_2$ oxidation of the ascorbate in a reaction catalyzed by HRP.
0.3 mm Diameter Flexible Amperometric Lactate Probe

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Abstract:

A flexible lactate electrode was made of 400±100 7 μm diameter carbon fibers, epoxy embedded in a 0.3 mm diameter polyimide tubing. The electrode was modified by precipitating on it the relatively insoluble complex formed between 1100 kd partially N-ethylamine quaternized poly[(vinylpyridine)Ost(bipyridine)2Cl] Cl (POs-EA) and lactate oxidase. The steady-state lactate electrooxidation current, at 2mM lactate concentration and at 22°C, was 400nA. The 50±0 μAcm⁻² current density and the 20mAcm⁻²M⁻¹ sensitivity decreased only by 5% upon increasing the partial pressure of oxygen from 0.0 to 0.2 atm. The electrode retains its sensitivity after dry storage at 4°C for 4 months in air but loses at 37°C half of its sensitivity in 7 hours through polymer desorption when operated at 0.4V (SCE). To eliminate interference by species that are electrooxidized at 0.4V (SCE), the lactate sensing probe was (a) electrically insulated with an epoxy made of poly(vinylimidazole) crosslinked with ethylene glycol diglycidyl ether, and (b) coated with an immobilized horseradish peroxidase (HRP)-glucose oxidase (GOX) film. The latter film was formed by co-immobilizing the two enzymes through periodate oxidation of their oligosaccharides to aldehydes and forming
Schiffs-bases between the polyaldehydes and the enzymes' lysyl amines. In the presence of 1 mM glucose and in air, the interfering electrooxidation of 0.1 mM ascorbate was reduced by a factor of 20. This reduction results from formation of hydrogen peroxide in the glucose catalyzed reaction, and \( \text{H}_2\text{O}_2 \) oxidation of the ascorbate in a reaction catalyzed by HRP.

**Introduction**

Simple glucose electrodes have been made by adsorbing a poly(\((\text{vinylpyridine})_2\text{bipyridine})\text{Cl}) \text{Cl} \) derivative on graphite, rinsing, then complexing glucose oxidase to the adsorbed polymer. Upon complexing, the adsorbed redox polymer binds and penetrates the enzyme and establishes electrical contact between the redox centers of the enzyme and a graphite electrode. Here we apply such a complexing process to forming lactate electrodes on flexible bundles of epoxy-embedded polyimide tubing-contained carbon fibers. The electrodes are coated with an interferant eliminating layer, containing coimmobilized horseradish peroxidase (HRP) and glucose oxidase (GOX). In an aerated glucose solution \( \text{H}_2\text{O}_2 \) is generated within the film through the GOX catalyzed reaction. The \( \text{H}_2\text{O}_2 \) oxidizes the interferants, but not lactate, in the HRP catalyzed reaction. The interference elimination process is built on that described for glucose electrodes, where lactate oxidase has been used to generate \( \text{H}_2\text{O}_2 \) in a lactate containing aerated solution.

Lactate oxidase from *Pediococcus* sp. commonly used in amperometric lactate sensors has been used throughout this work. The
enzyme catalyzes reaction 1 and also the oxidation of the FADH₂ by O₂, whereby H₂O₂ is formed.

\[
\text{LOX-FAD + Lactate} \rightarrow \text{Pyruvate + LOX-FADH}_2
\]  \hspace{1cm} (1)

Amperometric lactate sensors have been based on this reaction combined with reactions 2 and 3:

\[
\text{LOX-FADH}_2 + 2M_{ox} \rightarrow \text{LOX-FAD - 2M}_{re}
\]  \hspace{1cm} (2)

\[
M_{re} \rightarrow M_{ox} + 2e^-
\]  \hspace{1cm} (3)

where \(M_{ox}\) and \(M_{re}\) are the oxidized and the reduced forms of a diffusional mediator, such as \(O_2\cdot H_2O_2\) or \(Fe^-/Fe\) (where Fe is a ferrocene derivative). FAD/FADH₂ centers of LOX were also non-diffusionally electrically connected to electrodes through a 3-dimensional redox epoxy-based electron relaying network.

Lactate mono-oxygenase from *Mycobacterium smegmatis* having an FMN co-factor, has also been applied in lactate sensors. This enzyme catalyzes the oxidation of lactate by oxygen to acetic acid and carbon dioxide. However, the enzyme is inhibited by phosphate, an ion present in blood and other tissues, whereas lactate oxidase from *Pediococcus* sp. is not inhibited by phosphate. Because the sensor is to be used in phosphate-containing physiological solutions, the *Pediococcus* enzyme was used in this work.
In vivo measurement of lactate is of clinical interest in monitoring shock, respiratory insufficiency and heart failure. Miniaturization and flexibility of sensors is relevant to lactate monitoring at a specific site, or in an organ where tissue damage upon electrode insertion must be avoided. Miniature glucose sensors based on glucose oxidase, an enzyme of higher specific activity than that of LOX, have been reported. One limit imposed on miniaturization is defined by (a) the achievable current density, which in turn can be limited by the specific activity of the enzyme in catalyzing reaction 1; or (b) the electron transfer rate to a diffusional mediator or polymer bound relay (reaction 2); or (c) the rate of mediator diffusion in solution or electron diffusion through the redox conducting polymer, that may limit the rate of reaction 3.

The redox polymer PO-EA forms relatively insoluble adducts with glucose oxidase and other enzymes, that can be crosslinked with diepoxides. The electronic conduction in the resulting redox epoxy network in glucose microelectrodes is sufficient to allow a current density in excess of 0.5 mA cm\(^{-2}\) and a sensitivity in excess of 50 mA cm\(^{-2}\) M\(^{-1}\). The glucose microelectrodes are relatively insensitive (\(\approx 3\%\)) to aeration and deaeration.

In order to provide strength, flexibility and adequate currents, so that Faraday cages will not be needed, carbon fiber bundles consisting of several hundred carbon fibers were used. A flexible polyimide tubing provided insulation.
Experimental:

Chemicals. Lactate oxidase (LOX, 35 units/mg) from *Pediococcus sp.* (EC number is not available) was purchased from Genencor Inc. and was used as received. Glucose oxidase (GOX, EC 1.1.3.4, type X, 128 units/mg), horseradish peroxidase (HRP, EC 1.11.1.7, type VI, 260 units/mg), L-lactic acid and ethylene glycol diglycidyl ether (EG) were from Sigma and were used as received. The redox polymer, POs-EA was synthesized as previously reported. Poly(vinylimidazole) (PVI) was synthesized as described.

Apparatus. An EG&G Princeton Applied Research 273 potentiostat/galvanostat and an Emman Instrumentation potentiostat with a conventional electrochemical cell were used respectively for the cyclic voltammetry and for the constant-potential experiments. All potentials are referenced to the cell's saturated calomel electrode (SCE).

A home-built flow cell built with a rotary injection valve (Beckman) was used for the flow injection analyses. The microelectrode was placed approximately 0.5 cm from the injection valve and the reference electrode about 1 cm from the working electrode.

Lactate microelectrode fabrication. The fiber bundle was made as follows: 300 to 500 carbon fibers were inserted into a 0.3 mm diameter 7 cm long polyimide tubing. A 0.1 mm polyimide insulated platinum wire, auxiliary electrode, was also inserted into the tubing. The tubing was
completely filled by capillary action with an epoxy "Super-Low Viscosity Embedding Media" (Polysciences, Inc., Warrington, PA). The fiber-epoxy composite was cured in the tubing at 80°C for 2 days, then glued to a stainless steel wire with an electrically conducting silver epoxy. An approximately 45° beveled electrode tip was formed by polishing sequentially with three grades of diamond paste (15, 6 and 1 micron), sonicating and washing with alcohol and distilled water. A drop of 1% Triton X-100 was then placed on the cleaned bevel. After air drying (~1h, 22°C), the surface was washed with deionized water. The purpose of the Triton X-100 treatment was to convert the hydrophobic epoxy surface to a hydrophilic for improved adsorption of the redox polymer and its adduct with LOX.

The surface area of the electrode was determined as follows. A 3Å thick gold layer was sputter-deposited on the beveled electrode tip, i.e., on the tops of the fibers and on the epoxy between the fibers. Next the currents of the gold coated electrode and of an exposed 2mm diameter gold disk electrode were determined in 0.5mM ferrocenemethanol and measured by differential polarographic voltammetry (DPV). The electrode surface area was then calculated from the ratio of the currents. The actual surface area of the epoxy-embedded fiber bundle was 0.009cm² ± 0.002cm², nearly an order of magnitude higher than the geometric area, showing that the surface was rough. Electron microscopy showed that the fiber tips protruded as hemispherical domes, from the embedding epoxy.

The lactate sensing electrode was formed by adsorbing the adduct of the redox polymer and lactate oxidase. The adsorption process was the
following: One droplet (0.2 to 0.3 μL) of a 10 μg/μL solution in 0.02M HEPES buffer pH 7.0 was transferred from the tip of a capillary tube onto the bevelled surface, first of the polymer, then of the enzyme. The mixed solution was allowed to dry under air for > 3 days. Coulometry showed an osmium loading of 45 ± 2mC cm⁻² based on the electrochemically estimated 0.009 cm² surface area. No diepoxide or other crosslinker¹⁹ was used in making these electrodes, i.e. functionalization depended solely on adsorption of the precipitated POs-NH₂-LOX complex. Before testing, the electrodes were soaked in 0.02M pH 7.01 sodium phosphate buffer containing 0.1M NaCl for at least three hours to remove leachable components.

The interferant eliminating layer was applied on top of a PVI-EG electrically insulating "barrier" layer, formed over the lactate sensing layer by an earlier described method.³ The PVI-EG layer was then coated by applying a 0.2 or 0.3μL droplet of a dissolved mixture of polyaldehydic HRP and GOX.³ This mixture was prepared as follows: 1mg HRP and 1mg GOX were dissolved in 50μL of 0.1M sodium bicarbonate, to which 5μL of sodium periodate (12mg/mL) was added.²⁵ The solution was incubated in the dark, at room temperature, for 7-8 hours. After incubation, the solution was dialyzed against 0.02M, pH 7.01 sodium phosphate buffer containing 0.1M NaCl in order to eliminate the excess periodate. The polyaldehydic HRP and GOD were allowed to self-crosslink by Schiff-base formation between the HRP aldehydes and GOX lysyl amines for two days, at 22°C.
All electrochemical experiments were carried out in 5 to 25mL stirred 0.02M, pH 7.01 sodium phosphate buffer containing 0.1M NaCl unless otherwise stated.

Results and Discussion:

Figure 1 shows the dependence of the logarithm of the anodic peak current (i_p) on the logarithm of the scan rate (SR) in cyclic voltammograms for the POs-EA polymer (but not gold coated carbon fiber-bundle electrode in 0.02M phosphate buffer with 0.1M NaCl). The slope is 0.54, near the theoretical 0.50 slope characteristic of a semi-infinite planar diffusion limited electrode. Evidently the fibers are too closely spaced for radial diffusion. With the POs-EA/LOX adduct precipitated on the fiber bundle the dependence of log i_p on log SR is also linear. For a single fiber microelectrode (7μm diameter), it has been proposed that superior sensor characteristics result from radial electron diffusion through the enzyme and redox polymer containing gel.14 Such characteristics are obviously not realized in the fiber bundle.

Figure 2 shows the doubling of i_p in peaks of the cyclic voltammogram (of a- cis-r-is(2, 2(4-methylamino)bipyridine-N,N') dichloroosmium(II) solution) upon Triton-X-100 treatment of the electrode. Evidently adsorption of the non-ionic detergent Triton X100 improves the wetting of the surface by the solution. Figure 3 shows cyclic voltammograms of the electrode without lactate and with 2mM lactate. In the absence of lactate the separation of the anodic and cathodic peaks is
40 ± 20mV. An increase in peak separation with scan rate is observed in thicker films, produced by crosslinking the enzyme polymer complex with polyethylene glycol diglycidyl ether. For a fast, strongly adsorbed redox couple, the separation would have been nil, while for a non-adsorbed fast redox couple the separation would have been 59mV.

Figure 3 shows cyclic voltammograms of the carbon fiber-bundle electrode without lactate and with 2mM lactate. In the absence of lactate, the separation of the anodic and the cathodic peaks is 40 ± 20mV. Maintenance of the electrode at -0.4V (SCE) for a prolonged period leads to loss of non-crosslinked redox polymer. This is evident from the reduction in the coulometrically measured charge associated with electroreduction and electrooxidation of the polymer. When 2mM lactate is added to the solution, only an electrocatalytic oxidation current is observed at scan rates below 5mVs⁻¹. In the absence of a crosslinker, the POs-EA/LOX layer thickness estimated by Os²⁻₃⁻ coulometry is 0.5 ± 0.2μm (assuming that the molecular weight of each Os containing segment is 1100 daltons and that the polymer's density is near 1.5 g cm⁻³). When the POs-EA complexes the enzyme, precipitation is observed at 1:1 and 1:3 polymer to enzyme ratios. Thus, the film formed on the fiber-bundle electrode contains a precipitate. Measurement of the oxygen sensitivity of the lactate fiber-bundle electrode made with a 1:1 polymer:enzyme weight-ratio film (Figure 4) shows that the steady state electrocatalytic current in air is only 3.6% below that in nitrogen at the physiologically relevant 1.5 mM lactate concentration.
The 10% to 90% risetime of the steady-state electrocatalytic current in a flow system at a 4 cm s\(^{-1}\) linear flow velocity (for an increase in lactate concentration from 0 to 2 mM, or 0 to 3 mM, or 0 to 4 mM) is 12 seconds.

The pH dependence of the electrocatalytic current was measured in air at 1 mM lactate concentration and at a POs-EA/LOX - 3:1 weight ratio. The current is constant within ±7% when the pH is varied between 6.4-8.5, the maximum being near pH 7.4 (figure 4). The pH dependence of the current exhibits a broader pH optimum and a shift to higher pH relative to the natural reaction of the enzyme producing H\(_2\)O\(_2\) by O\(_2\) reduction. In an earlier reported macroelectrode with crosslinked lactate oxidase-redox polymer network, the current was also within 20% of its maximum through the same 6.4-8.5 pH range. The current maximum was, however, at pH 8, not 7.4. This small but significant difference suggests that the precipitated POs-EA:LOX film and the diepoxide crosslinked film do not provide identical enzyme microenvironments.

As seen in Table 1, an electrode stored at 4°C for 4 months did not lose activity. In continuous operation of the lactate electrode at 0.45V (SCE) and at 25°C, the current drops in 6 hours to 75% of its initial value (Figure 6), primarily because of polymer desorption at positive potentials, where the polycation is highly charged. This desorption is evidenced by the reduced coulometrically measured charge upon electroreduction or electrooxidation of the adsorbate in the absence of lactate.
Figure 7 illustrates the electrode structure with lactate sensing and interference eliminating layers. As in glucose sensors, oxidizable species present in physiological samples (serum or blood), such as ascorbate, urate or acetaminophen (Tylenol) interfere with the sensing of lactate. An interferant eliminating layer preoxidizes the interferants on their way to the sensing layer through reactions 4 and 5.

\[
\text{GOX} \\
\text{Glucose} + \text{O}_2 \quad \text{-----} \quad \beta\text{-gluconolactone} + \text{H}_2\text{O}_2 \quad (4)
\]

\[
\text{HRP} \\
\text{H}_2\text{O}_2 + 2\text{HA} \quad \text{-----} \quad 2\text{A} + 2\text{H}_2\text{O} \quad (5)
\]

In equation 5 HA is an interfering hydrogen donor. The physiological glucose concentration in serum or in blood (3-10mM) generates enough hydrogen peroxide to preoxidize all interferants in the presence of HRP, and the enzyme catalyzed preoxidation of interfering hydrogen donors is fast enough to prevent the interferants from reaching the lactate sensing layer. In the structure shown in Figure 7, an electrically insulating barrier layer prevented electron transfer to oxidized HRP from the LOX-wiring redox polymer. The insulating layer was thick enough to prevent electrical communication between the sensing and the interferant eliminating layers yet thin enough so as not to hinder substrate or product diffusion.

Figure 8 shows calibration curves for HRP-GOX coated interference eliminating electrodes made with and without electrically insulating
PVI/EG barrier layers between the sensing and interference eliminating layers. The barrier layer reduced the current by approximately 40%, and increased the 10-90% risetime from 12s to about 1 min. The left side of Figure 9 shows the reduction of interference by ascorbate (0.1mM). When glucose (1mM) was added, about 95% of the ascorbate related current was eliminated. The right side of the figure shows the increase in current upon increasing stepwise the lactate concentration from 0 to 2mM. Because lactate was not oxidized by H2O2-HRP, the electrode continued to respond normally to lactate. Figure 10 shows lactate calibration curves with and without 0.1mM ascorbate. The current measured with 0.1mM ascorbate was about 7.5% higher than that with no ascorbate. Part of the increase resulted from the reduction in oxygen partial pressure, caused by consumption of oxygen in the oxidation of glucose in the outer layer. The observed 7.5% current increase was, however, characteristic only of the 3:1 enzyme-polymer film electrode, where oxygen competition was greater than in the 1:1 electrode. In the 1:1 electrode oxygen competition suppressed the current by only 3.6%, and the ascorbate elimination related current increase was correspondingly smaller. Earlier we showed that in glucose sensors the combination of all interferants (ascorbate, urate and acetaminophen) at their physiological levels was preoxidized in interference eliminating LOX-HRP films. The physiological lactate concentrations (1-3mM) are lower than physiological glucose concentrations (3-10mM) and glucose oxidase activity is higher than lactate oxidase activity in the films. Therefore, we project that at physiological glucose concentrations the entire group of electrooxidizable interferants with lactate measurements will be effectively preoxidized.
After immobilizing its components and over-coating with a bioinert film, the electrode should be practical for whole-blood lactate assay and for implantation.

Acknowledgements: Parts of the work were supported by the Office of Naval Research, National Institutes of Health under Grant No. IROI DK42015-01A1, National Science Foundation, and the Robert A. Welch Foundation.

References


Figure 1 Dependence of the logarithm of the anodic peak current on the logarithm of the scan rate for an 0.3 mm carbon fiber-bundle electrode on which the POs-EA redox polymer was adsorbed.

Figure 2 Cyclic voltammograms of 0.5mM cis-bis(2, 2' (4-methylamino) bipyridine-N,N'dichlorosmium(II) on a carbon fiber-bundle electrode in pH 7.13 phosphate buffer with 0.1M NaCl at 10V/s scan rate. (●) Without Triton X-100 treatment of the electrode; (■) With Triton X-100 treatment of the electrode.

Figure 3 Cyclic voltammograms of the lactate electrode in pH 7.13 phosphate buffer with 0.1M NaCl, under air. Scan rate: 2 mV/s. a) no lactate, b) 2.0mM lactate.

Figure 4 Steady-state lactate response of the electrode at 0.4V (SCE), under nitrogen and air: 0.02M phosphate with 0.1M NaCl; pH 7.13; room temperature. Film composition: POs-EA : LOX = 3:1 (weight ratio)

Figure 5 pH dependence of LOX's activity. (●) LOX in solution, H₂O₂ generation [27] and (■) LOX immobilized on the electrode. 1mM lactate, 0.02M phosphate with 0.1M NaCl, pH 7.13, in air, room temperature.

Figure 6 Decay of the steady-state current of the electrode coated with POs-EA : LOX (1:1 weight ratio). 2mM lactate; Conditions as in Fig. 5.
**Figure 7** Schematic drawing of the lactate sensor showing the insulating barrier and the interferant eliminating overlayer.

**Figure 8** Steady-state lactate response curves for electrodes with (●) and without (□) barrier layers. Conditions as in Fig. 5.

**Figure 9** Reduction of ascorbate current by an HRP-GOX film. Conditions as in Fig. 5.

**Figure 10** Steady-state lactate current without (□) and with (●) 0.1mM ascorbate in the solution. Conditions as in Fig. 5.
Interferant eliminating layer (HRP/GOX)

Barrier layer (PVI/EG)

Lactate sensing layer (POs-NH₂/ LOX)

Epoxy embedded carbon fiber array in polyimide tubing
Table 1  Storage stability at 4°C in air

<table>
<thead>
<tr>
<th>Days</th>
<th>Current (nA) at 2mM lactate</th>
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<tbody>
<tr>
<td>0</td>
<td>11.1</td>
</tr>
<tr>
<td>30</td>
<td>10.5</td>
</tr>
<tr>
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<td>11.6</td>
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<tr>
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</tr>
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