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[54] CONVENIENT DETERMINATION OF TRACE LEAD IN WHOLE BLOOD AND OTHER FLUIDS

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[21] Appl. No.: **821,732**

[22] Filed: **Jan. 15, 1992**

[51] **Int. Cl.**[5] **G01N 27/26**

[52] **U.S. Cl.** **204/403**; 204/412; 204/415; 204/435; 435/817; 435/288

[58] **Field of Search** 204/403, 412, 415, 435; 435/288, 817

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,581,336	4/1986	Malloy et al.	204/403
4,820,399	4/1989	Senda et al.	204/403
4,950,378	8/1990	Nagata	204/412
4,970,145	11/1990	Bennett et al.	204/403

OTHER PUBLICATIONS

Crumbliss, A. L., Henkens, R. W., Kitchell, B. S., Perine, S. C., Stonehuerner, J., and Tubergen, K. R., "Amperometric Glucose Sensor Fabricated from Glucose Oxidase and a Mediator Co-Immobilized on a Colloidal Gold Hydrogel Electrode," *Biosensors Technology: Fundamentals and Application,* Chapter, 13, 1990, published in USA.

Crumbliss, A. L., Henkens, R. W., Hunter, K., Kitchell, B. S., O'Daley, J. P., Stonehuerner, J., and Tubergen, K. R., "The Influence of Colloidal Gold Surfaces on Enzyme Activity," ACS North Carolina Divisional Meeting, Sep. 1988, published in USA.

Crumbliss, A. L., Henkens, R. W., Kitchell, B. S., Perine, S. C., Stonehuerner, J., and Tubergen, K. R., "Amperometric Glucose Sensor Fabricated from Glucose Oxidase and a Mediator Co-Immobilized on a Colloidal Gold Hydrogel Electrode," ACS North Carolina Divisional Meeting, University of North Carolina at Chapel Hill, Sep. 7-9, 1989, published in USA.

Crumbliss, A. L., Kitchell, B. S., Perine, S. C., Stonehuerner, J., Tubergen, K. R., Zhao, J., and Henkens, R. W., "Catalytic and Electroactivity of Irreversibly Adsorbed Enzymes at Gold Electrode Surfaces," Symposium on Protein Electrochemistry: ACS Southeast Regional Meeting (SERM), Oct. 1989, published in USA.

Crumbliss, A. L., Henkens, R. W., Kitchell, B. S., McLachlan, K. L., O'Daly, J. P., Perine, S. C., Stonehuerner, J., Tubergen, K. R., and Zhao, J., "The Use of Inorganic Materials to Control or Maintain Immobilized Enzyme Activity," Symposium on opportunities for inorganic chemistry in biotechnology, ACS National Meeting in Boston, Apr. 23, 1990, published in USA.

Henkens, R. W., Kitchell, B. S., O'Daly, J. P., Perine, S. C., and Crumbliss, A. L., "Bioactive Electrodes Using Metallo Proteins Attached to Colloidal Gold," *Recl.: Trav. Chim. Pays Bas,* 106:298, 1987.

Henkens, R. W., Zhao, J., and O'Daly, J. P., "Multi--Analyte Enzyme Electrodes for Environmental Monitoring," *Proceedings of 5th International Biotechnology Conference in Copenhagen,* Jul. 8-13, 1990.

Albery et al., "Inhibited Enzyme Electrodes. Part 3.," *Biosensors & Bioelectronics,* 5:397-413, 1990, published in Great Britain.

(List continued on next page.)

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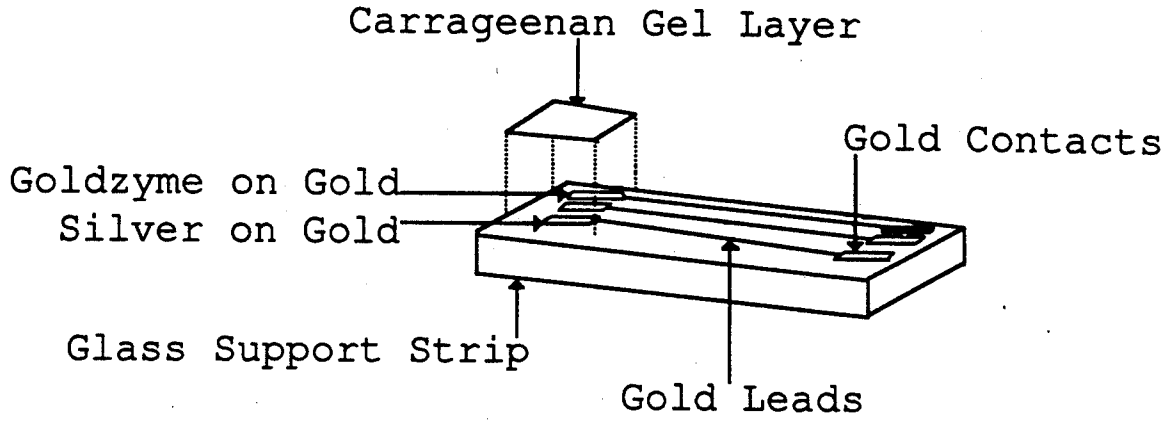
[57] **ABSTRACT**

The invention relates to novel b of detecting metal ion concentrations less than about 10 μg/dl fluid. Biosensors based on the enzyme isocitrate dehydrogenase are particularly suited for detecting trace lead ion concentrations in water and in blood. Bioelectrodes are fabricated from surface deposited colloidal gold adsorbed enzyme that retains high catalytic activity Other aspects of the invention include detection devices for convenient and rapid measurement of metal ions.

30 Claims, 7 Drawing Sheets



OTHER PUBLICATIONS

Almestrand et al., "Determination of Lead in Whole Blood with a Simple Flow-Injection System and Computerized Stripping Potentiometry," *Analytica Chimica Acta*, 209:339–343, 1988, published in The Netherlands.

Baum & Czok, "Enzymatische Bestimmung vol „ ionisiertem++ Magnesium in Plasma," *Biochemische Zeitschrift*, 332:121–130, 1959.

Botré et al., "Synthesis and Inhibitory Activity on Carbonic Anhydrase of Some New Sulpiride Analogues Studied by Means of a New Method," *Journal of Medicinal Chemistry*, 29:1814–1020, 1986.

"New Rules Set for Blood Lead Levels," *Chemical and Engineering News*, p. 17, Oct. 14, 1991.

Fair & Jamieson, "Studies of Protein Adsorption on Polystyrene Latex Surfaces," *Journal of Colloid and Interface Science*, 77(2):525–534, 1980.

Guilbault et al., "Homovanillic Acid as a Fluorometric Substrate for Oxidative Enzymes," *Analytical Chemistry*, 40(1):190–196, 1969.

Guilbault, "Determination of Inhibitors," *Enzymatic Methods of Analysis*, Pergamon Press, pp. 197–209, 1970, published in Great Britain.

Holleck, "The Reduction of Chlorine on Carbon in $AlCl_3$–KCl, NaCl Melts," *Journal of the Electrochemical Society*, 119(9):1158–1161.

"U.S. CDC Releases Revised Guidelines on Childhood Lead Poisoning-Blood Lead Level of Concern Lowered to $\geqq 10$ μg/dl," *ILZRO Environmental Update*, 1(10):2, 1991.

Kamata & Onoyama, "Lead-Selective Membrane Electrode Using Methylene Bis(diisobutyldithiocarbamate) Neutral Carrier," *Analytical Chemistry*, 63:1295–1298, 1991.

Kratochvil et al., "Effect of Metals on the Activation and Inhibition of Isocitric Dehydrogenase," *Analytical Chemistry*, 39(1):45–51, 1967.

Linde, "Estimation of Small Amounts of Fluoride in Body Fluids," *Analytical Chemistry*, 31(12):2092–2094, 1959.

Morrissey & Han, "The Conformation of γ-Gluobulin Adsorbed on Polystyrene Latices Determined by Quasielastic Light Scattering," *Journal of Colloid and Interface Science*, 65(3):423–431, 1978.

Sheikh & Townsend, "Aplications of Enzyme-Catalysed Reactions in Trace Analysis-VII," *Talanta*, 21:401–409, 1974, published in Great Britian.

Smit & Cass, "Cyanide Detection Using a Substrate-Regenerating, Peroxidase-Based Biosensor," *Analytical Chemistry*, 62:2429–2436, 1990.

Toren & Burger, "Trace Determination of Metal Ion Inhibitors of the Glucose-Glucose Oxidase System," *Mikrochimica Acta* (*Wien*), pp. 538–545, 1968.

Tran-Minh et al., "Studies on Acetylcholine Sensor and its Analytical Application Based on the Inhibition of Cholinesterase," *Biosensors & Bioelectronics*, 5:461–471, 1990.

Smith, "Air Pollution and Forest Damage," *Chemical and Engineering News*, pp. 30–42, Nov. 11, 1991.

Trade Brochure: esa, Inc. Trace Metal Analyzer Brochure, Bedford, Mass., A, 1990.

Gunasingham et al., "Performance and Evaluation of a Handheld Electrochemical Monitor for Toxic Metals," Cole-Parmer Instrument Company, Chciago, Illinois.

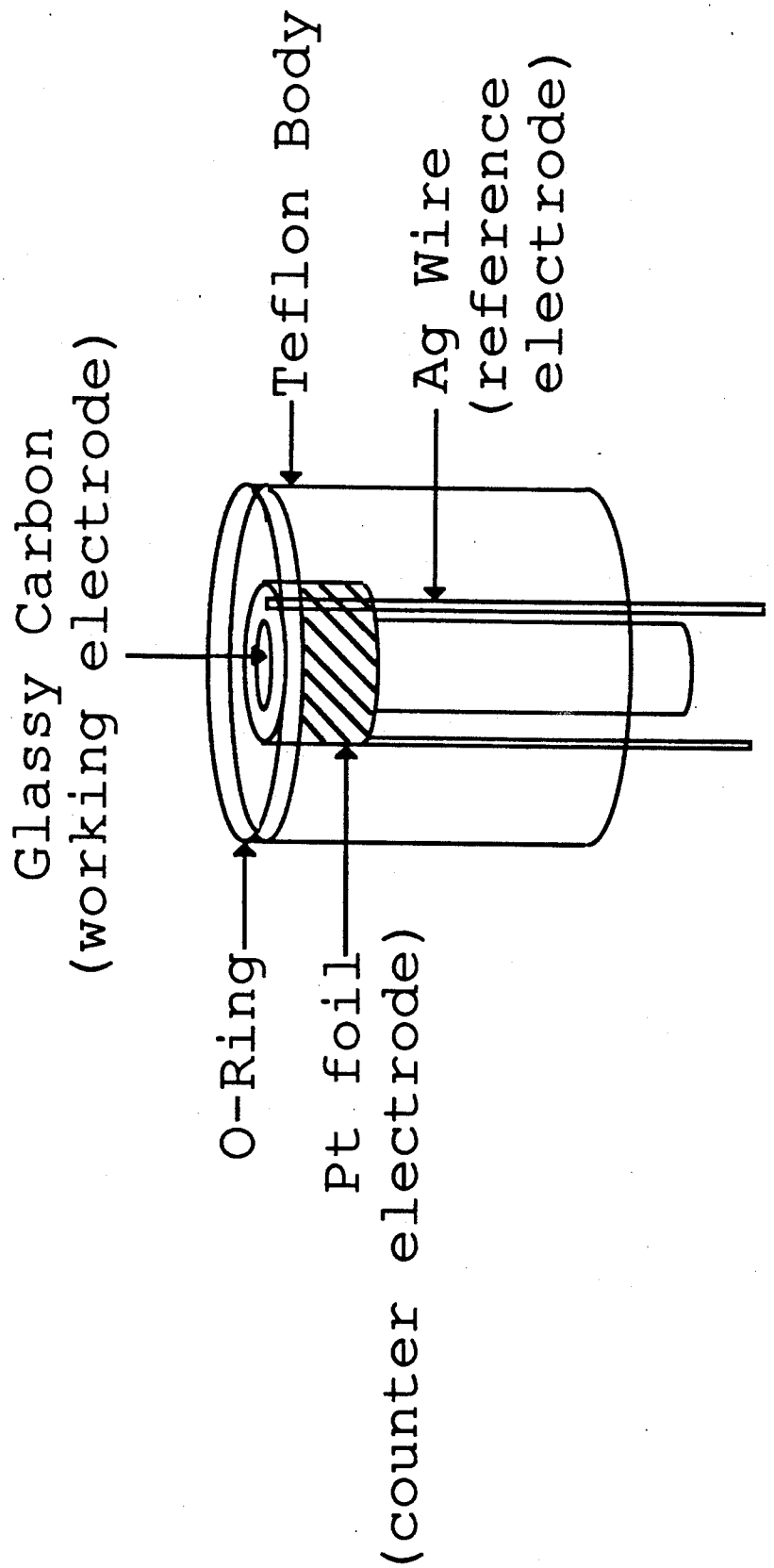

Glassy Carbon
(working electrode)
Teflon Body
Ag Wire
(reference
electrode)
O-Ring
Pt foil
(counter electrode)

FIGURE 1

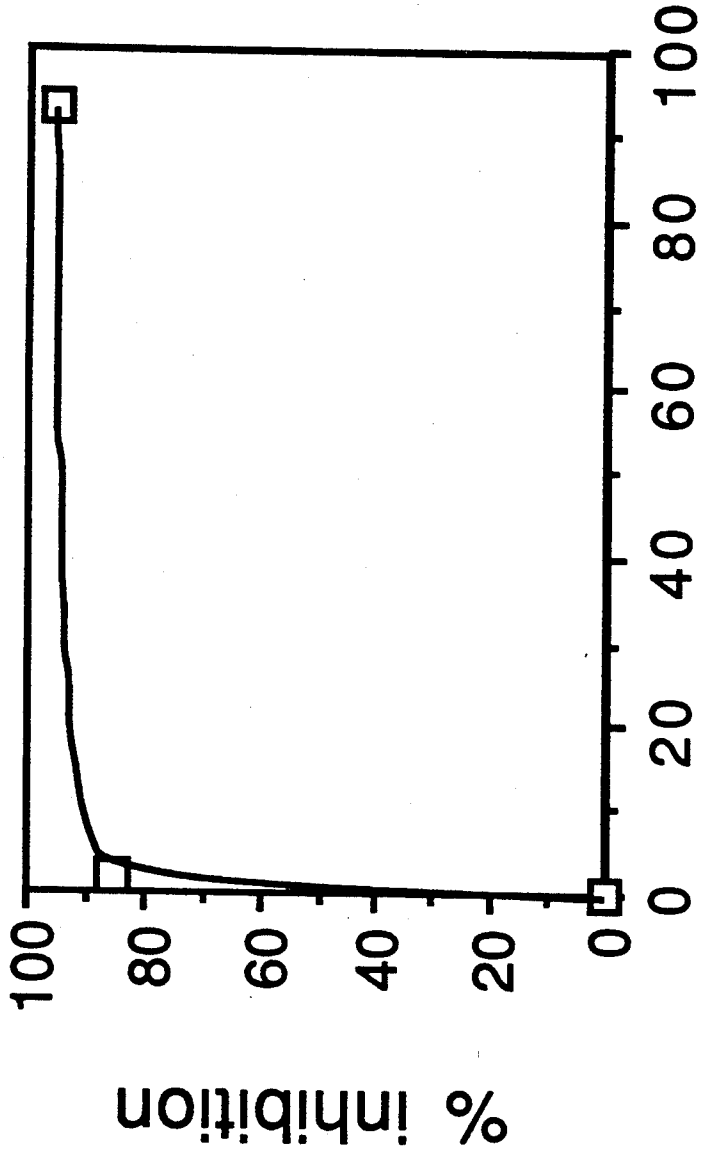


FIGURE 2

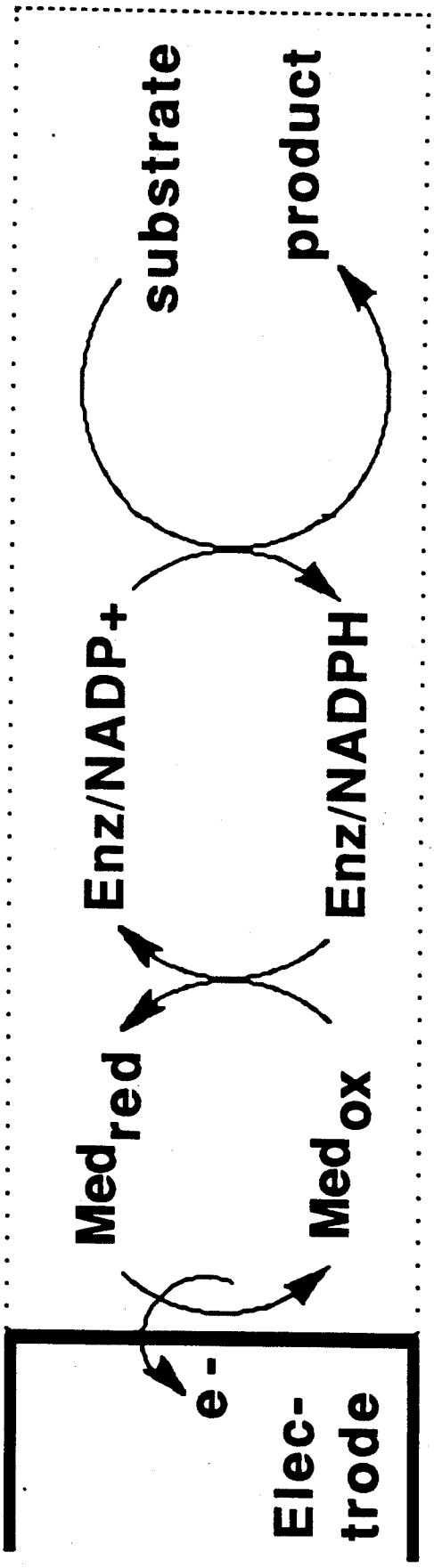

substrate
product
Enz/NADP+
Enz/NADPH
Med red
Med ox
e-
Elec-
trode

FIGURE 3

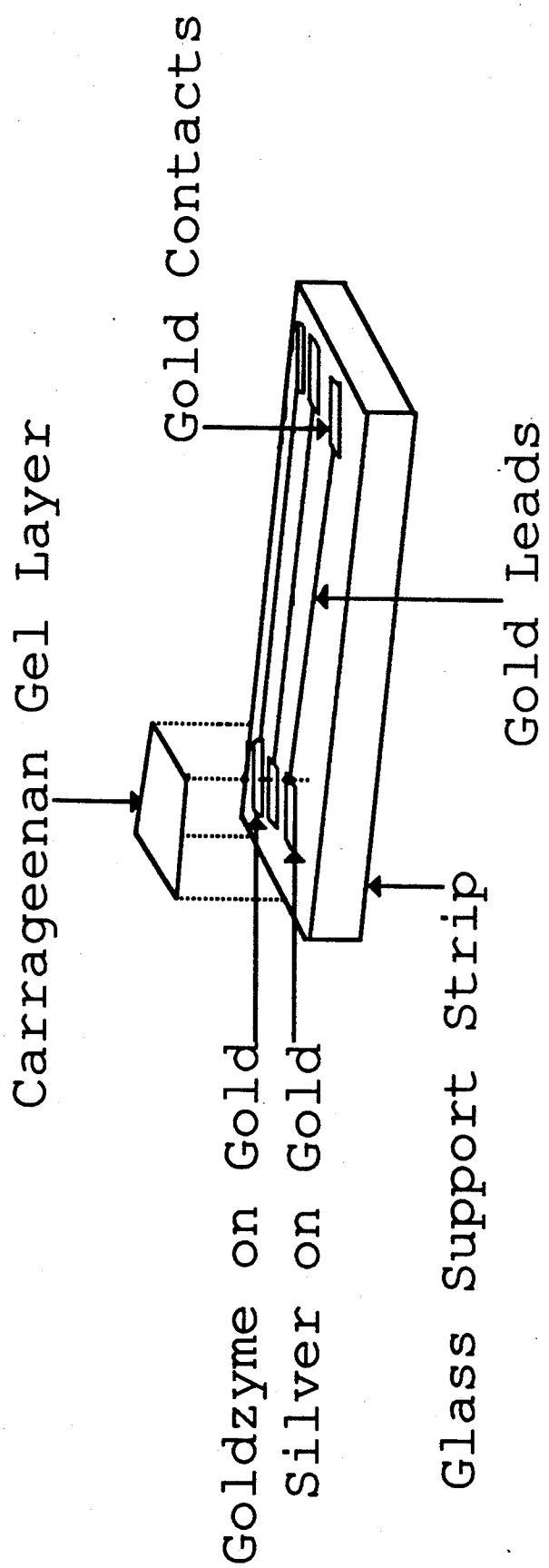

Gold Contacts
Carrageenan Gel Layer
Gold Leads
Goldzyme on Gold
Silver on Gold
Glass Support Strip

FIGURE 4

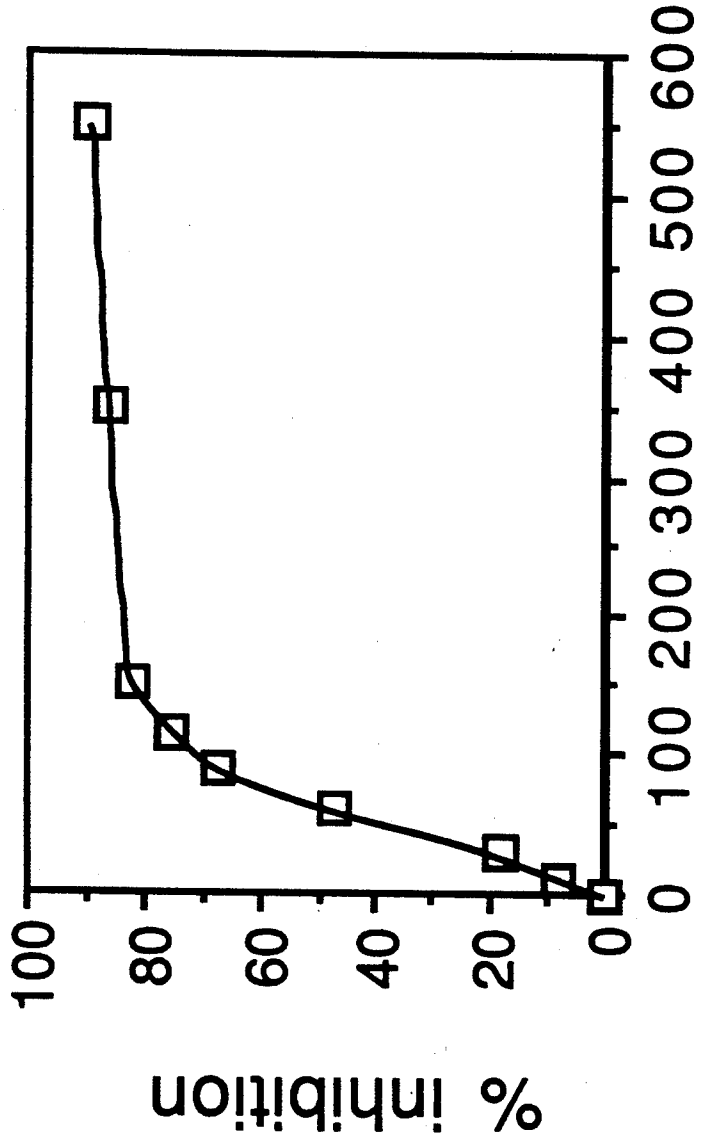


FIGURE 5

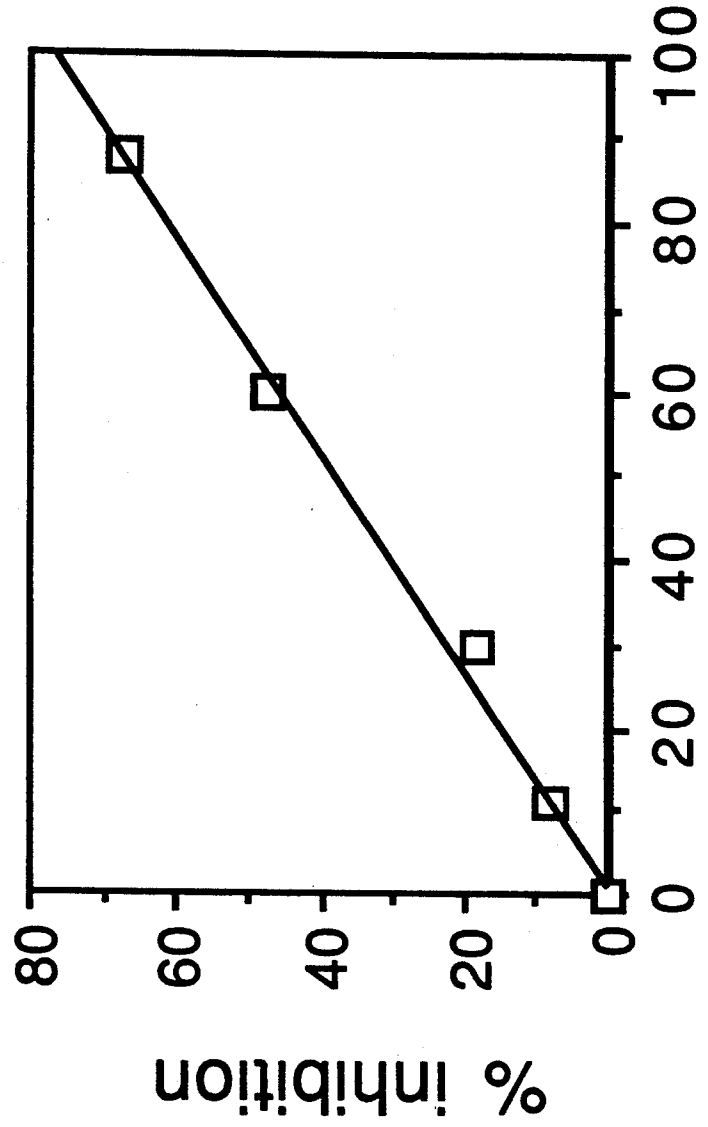


FIGURE 6

$$\text{isocitrate} + \text{NAD}+ \longrightarrow \alpha\text{-ketoglutarate} + \text{NADH} + \text{H}^+$$

$$\text{NADH} + 2\,\text{MED}_{ox} \longrightarrow \text{NAD}+ + 2\,\text{MED}_{red} + \text{H}^+$$

$$2\,\text{MED}_{red} - \text{e}^- \longrightarrow 2\,\text{MED}_{ox}\ \text{(oxidation current)}$$

Current may be detected directly at the electrode surface during oxidation of the cofactor, either directly or through a mediator.

Pig heart NADP-linked isocitric dehydrogenase (ICD, EC 1.1.1.42), an oxidoreductase, is sensitive to trace level of lead ion as low as 1 μg/dl. Like many enzymes, ICD is inactive or only slightly active unless Mg^{++} or Mn^{++} is present. In fact, at low concentrations of activator, e.g., <200 ppb Mn^{++}, the activity of ICD is proportional to Mn^{++} and may be used to determine activator concentration (Guilbault, 1970). Inactivation by inhibitors may also be used to determine inhibitor concentration. Specificity of enzymes for activators, or inhibitors, is not as great as for the natural substrate of the enzyme, but in some cases inhibitor concentration may be selectively determined. Silver and mercury have been determined in the presence of each other using isocitrate dehydrogenase (Mealor and Townshend, 1968). However, until now, there has been no satisfactory rapid amperometric method to determine of lead ion based on ICD inhibition, much less a simple method to determine low lead ion concentrations in whole blood.

As used herein, a bioelectrode refers to a single electrode, the working electrode, at the surface of which an electron transfer takes place representing a reaction catalyzed by an enzyme located on or near the surface of the electrode. Such a bioelectrode when set up with an appropriate reference/counter electrode may constitute a biosensor. Within the meaning of the present invention, a biosensor is intended to indicate a system capable of producing a signal that may be related to a reaction catalyzed by an enzyme constituting the biosensor. Biosensors comprising bioelectrodes will operate by producing a current related to the activity of an enzyme catalyzing electron transfer.

Enzymes have been immobilized at surfaces, but a major problem has been to maintain catalytic activity of enzymes immobilized on surfaces. This is of particular concern when ability to transfer electrons is involved, as with redox enzymes and such species as oxidases and reductases. The present invention takes advantage of the ability of colloidal gold adsorbed enzymes to retain high activity. Additionally, the deposition of colloidal gold adsorbed enzyme onto a working electrode surface has provided a catalytically active enzyme from which a detectable current can be generated.

A colloidal gold surface is distinctly different from flat bulk gold. Although the exact nature of the colloidal gold/protein/electrode surface interaction is not completely understood, there are several properties of colloidal gold that may enhance electron transfer between a redox protein and an electrode surface. Colloidal gold particles have high surface to volume ratio with high surface energy. Uncontaminated gold sol surfaces are highly active and can interact strongly with protein molecules. Smaller colloidal gold particles, in the range of 30 nm, provide an adsorbed protein molecule some freedom in orientation thereby increasing the possibility that a prosthetic group, or cofactor, is closer to the metal particle surface. This makes the distance for electron transfer between the protein and the metal particles shorter, and therefore charge transfer is easier. With ICD/gold sol deposited on an electrode surface, colloidal gold particles covered with ICD are able to function as electron-conducting pathways between the prosthetic groups and the electrode surface to facilitate the electron transfer process.

The larger effective surface area appears to allow more enzyme molecules to be immobilized at or near the electrode surface. The possibility for multilayers of effective Au-enzyme layers may be another mechanism by which the signal from colloidal gold assisted immobilization is increased.

Assuming that the average diameter of the sol particle is 30 nm and the density is 17.0 g/mL, then 3 μl of 7.5 mg Au/mL sol deposited onto a glass carbon surface of 3 mm in diameter is equivalent to about 12 layers of Au sol particles. In the case of HRP-Au sol this surface coverage gives the best performance, both with and without a mediator. Addition of more Au layers causes the unmediated response to deteriorate slightly but does not much affect the mediated response, suggesting that the deposited Au layers are not very porous and that the accessible depth is no more than 12 layers of deposited Au sol. Even within the 12 layers, only the outermost layers are of importance, because changing from 4 to 12 layers increased the signal by only 10–20% with or without a mediator. This suggests that only a few layers of the deposited Au-enzyme are porous. In consideration of both the electrode performance and cost, 3 μl ICD-Au sol appears to be optimum for a 3 mm diameter glassy carbon surface.

Although apparently only the outermost gold layers contribute the major portion of accessible enzyme molecules, enzyme loading and mediator effects with colloidal gold assisted immobilization are still much higher when compared with simple adsorption on flat surfaces. Spectroscopic data for the enzymatic activity of some enzymes adsorbed on colloidal gold before deposition on the electrode surface indicate that the active enzyme coverage on the gold sol particle surfaces is only about 40% of a theoretical compact monolayer. This is consistent with the data obtained by other workers for adsorption of γ-globulin onto latex particles (Morrissey and Han, 1976; Fair and Jamieson, 1980). The likelihood of multilayer adsorption of protein molecules on a solid support surface is thought negligible. If the adsorption is not specific, the protein molecules can have multiple orientations on the surface. The strong interactions between the protein and the Au sol surface may increase the surface density of the adsorbed protein, and some of the restricted orientations may also favor the direct electron transfer between the protein molecules and the conductor surface. It is likely that all of the enzyme molecules are on the first layer of the adsorbed surface, but only part of the molecules have the correct orientation for direct electron transfer.

The electrode surface on which an enzyme/colloidal gold sol is deposited may be any appropriate conducting surface such as gold, platinum, glassy carbon and the like. In preparing lead sensitive electrodes with isocitric dehydrogenase, a glassy carbon surface is preferred.

Deposition of a lead-detecting enzyme on or near an electrode surface may be accomplished in several ways, including electrodeposition, evaporation, spray deposition (e.g., aerosol), or electrolyte deposition. Electrode-

position may be accomplished by setting a working electrode at an appropriate potential, for example 1.6 v vs. a Ag/AgCl reference electrode with a platinum wire counter electrode. Using a two electrode system with a glassy carbon disk electrode held at a fixed position in a cavity in a lucite block, or other suitable material, a platinum plate at the bottom of the cavity serves as a reference/counter electrode. Electrodeposition may be performed at constant current or constant potential and optimized for the enzyme to be deposited.

Electrolyte deposition is a method whereby an enzyme/Au sol is applied to the surface of an electrode such as glassy carbon followed by an equal volume of an electrolyte such as $CaCl_2$. The latter solution causes the sol to precipitate and after a period of time at ambient temperature the electrode may be rinsed and stored in buffer at 4° C.

Solvent evaporation is preferred for ease and convenience. The method is simply performed by applying a fixed amount of Au-enzyme sol to the electrode surface and then drying at room temperature or near 4° C.

Methods

A Pine Instrument dual potentiostat interfaced to an IBM-386 computer was for enzyme electrode measurement. The system is controlled with an ASYST program (J. Zhao, Enzyme Technology Research Group, Inc., 710 West Main Street, Durham, NC 27701).

Cyclic voltammetry measurements were used to determine amounts of immobilized mediator Cyclic voltammograms were obtained in the quiescent state. In steady state amperometry experiments the potential was set at 0 V/Ag in stirred buffer with regular sized cell or in quiescent solution with a micro cell and the steady state current was measured. A fixed potential method or chronoamperometric method was used to determine enzyme inhibition.

In the chronoammetry method, the working electrode was held at a fixed potential while current versus time data were collected with the aid of a computer until steady state was reached. This was observed either from a real-time graphic display and/or the numeric display on the computer screen. After measurement was complete, the computer was set to automatically provide a calibration curve of percentage inhibition vs. inhibitor concentration, heterogeneous binding constants for reversible inhibitors, and/or binding rate constants for irreversible inhibitors. Programs were modified as required.

In general, enzymes were purchased as indicated and used directly. Results were improved in some cases after the stock enzyme was purified by dialysis. Isocitrate dehydrogenase (Sigma, St. Louis, MO, ICD Type VI) was dialyzed against buffer containing buffer and manganese ion. If extensive dialysis was performed, substantial enzyme activity was lost; therefore, manganese ion was added to the dialysis buffer.

The following examples are intended to illustrate the practice of the present invention and are not intended to be limiting. Although the invention is demonstrated with isocitrate dehydrogenase and alcohol dehydrogenase, numerous variations of these enzymes are contemplated without changing the enzyme's susceptibility to irreversible inhibition by low metal concentrations. Likewise, other enzymes with different substrate specificity but similar selective sensitivity will also be appropriate.

EXAMPLE 1

The following example illustrates the detection of trace amounts of lead ion in aqueous medium through the inhibition of ICD in homogeneous solution.

Dialysis of ICD and Selection of Buffer

Commercially available ICD (Sigma Chemical Company, St. Louis, MO) typically contains considerable amounts of sulfate or EDTA that interfere with lead ion inhibition of ICD. Dialysis of ICD against low ionic strength phosphate buffer lost most of its activity which was restored by addition of Mn^{++}. Dialysis against Tris buffer containing a low concentration of Mn did not affect ICD activity.

Buffer selection was important because of potential interactions of the buffer with lead ion. Tris buffer did not cause interference, while carbonate or phosphate buffers were unsatisfactory because of lead ion interactions.

Mediator

NADPH was directly oxidized at high potentials (>0.7V vs. Ag/AgCl) on carbon electrode. However, at this high potential the background current was high. Additionally, the electrode surface was fouled, presumably due to polymerization during the oxidation process. Direct oxidation of NADPH produced a background current of approximately 900 nA while the total current with isocitrate was only about 1600 nA.

Ferricyanide produced a relatively high background signal because of operation at potentials >0.2V vs. Ag/AgCl. N-methylphenazine methosulfate produced a background signal of about 20 nA without isocitrate while the total signal with isocitrate was more than 500 nA at 0 V vs. Ag/AgCl.

Microcell

A glassy carbon rod of 3 mm diameter was wrapped in teflon tubing as working electrode and surrounded with a layer of Pt foil as the counter electrode with a silver wire placed in between as the reference electrode. At least one layer of teflon was inserted between two of the three electrodes. All three electrode surfaces were on the same plane. Tubing was fixed on the top of the coplanar electrode surface with an O-ring, forming a microcell of 100–200 μl in volume. Microcell configuration is shown in FIG. 1.

Measurement of Lead in Aqueous Solution

To the microcell was added in sequence: 100 μl 50 mM pH 8.5 Tris buffer, ca. 0.3–0.4 units ICD and, after 15 min, 5 μl 25 mM NMP-MS, 5 μl 60 mM NADP. The background current was measured at 0 V. 10 μl of 0.5 M isocitrate was added and the current measured again. The difference in the two signals was taken as due to the oxidation of isocitrate catalyzed by ICD.

To measure lead inhibition of ICD, lead ion was added to the above solution after addition of ICD but 15 min prior to the addition of NMP-MS and NADP. The current difference with isocitrate was due to inhibition of ICD by the added lead. Typical data are shown in Table 1. FIG. 2 indicates the sensitivity of the electrode to lead ion concentrations in the submicromolar range.

TABLE 1

$[Pb^{++}]/\mu M$	0	1.89	90.9

TABLE 1-continued

current/nA	1480	218	26
% inhibition	0	85.3	98.2

K_i=0.33 μM or 6.83 μg/dl lead

The presence of the mediator NMP-MS interfered with inhibition of the enzyme by added lead ion. The methosulfate group apparently caused the lead to precipitate.

EXAMPLE 2

The following example illustrates a typical preparation of an active enzyme adsorbed to colloidal gold. Such enzymes may be used to prepare bioelectrodes, generally by evaporative or electrodeposition of the enzyme/colloidal gold solution onto a suitable electrode surface.

Colloidal Gold Adsorbed ICD

Colloidal gold solutions were prepared by adding a solution of 1% aqueous sodium citrate to a boiling rapidly stirred solution of gold trichloride and refluxing for 30 min. Final concentrations (w/w) were 0.01% HAuC14 and 0.03% sodium citrate The particle size was estimated by filtration of the sol through polycarbonate membranes (Nuclepore Corporation, Pleasanton, CA) of varying pore size using an Amicon micro ultrafiltration unit Approximately 40% of the sol passed through a 500 A Nucleopore filter and was quantitatively collected on a 300 A Nucleopore filter.

The gold sol was concentrated by centrifugation at room temperature. The concentrated sol was mixed with appropriate amounts of dialyzed isocitrate dehydrogenase solutions Then a fixed amount of the Au-ICD sol was evaporated on a coplanar carbon electrode surface and the activity measured The ICD concentration profile in the Au-ICD sol was constructed vs. the measured immobilized activity to determine the optimum composition of the Au-ICD sol.

At low ICD loadings, the enzyme activity was too low to generate a detectable signal. As the loading increased the ICD-Au sol became unstable and precipitated At higher loadings the sol became stable and the immobilized ICD activity was good.

Electrochemical Measurement of Immobilized ICD Activity

After evaporation of ICD-Au sol onto a carbon electrode surface, the electrode surface exhibited a yellow-gold appearance which was not washed off. The electrode surface was briefly rinsed with water to remove any loosely bound material before measurements were made. Buffer solution with NMP-MS and NADP was added to the microcell. Background current was measured, then isocitrate added and current again measured Typical background and sample signals were 25 and 550 nA respectively.

The basic operational principle for ICD is shown in FIG. 2. An electron transfer mediator for efficient charge coupling with the electrode surface is required. When substrate concentration is sufficiently high, the generated oxidation current signals are directly proportional to the total amount of enzyme immobilized on the electrode surface. A mediator carries electrons between the enzyme(cofactor) and the electrode surface. Substrate is consumed with the production of a catalytic current.

EXAMPLE 3

The following example illustrates several methods contemplated for the detection of lead in whole blood using the bioelectrode of example 2. The addition of whole blood to the microcell of example 2, regardless of lead content, reduced current signal.

A bare coplanar carbon electrode was used to determine whether a current could be generated. In a microcell containing 0.1 ml Tris buffer with appropriate amounts of ICD, NMP-MS, NADP and isocitrate (see Example 1) the electrode produced an oxidation current at 0 V relative to Ag/AgCl.

Whole blood interfered with the electrode response to lead in a solution where ICD, NADP, NMP-MS, isocitrate, blood and buffer are mixed together. Presence of the blood increased the viscosity of the mixture and slows the diffusion process of molecules such as NADP (mw ca. 743) and NMP-MS which are fairly large and have only limited concentrations in practical usage. Several methods are envisioned to overcome this problem, including:

Co-immobilization of Key Elements

The interference arises from the low usable concentrations of ICD, NADP and NMP-MS. When diffusion processes are slow because of solution viscosity, the generated electric signal is reduced. If all key elements required for signal generation are immobilized on or near the electrode surface, long range diffusion or mass transfer is no longer necessary for signal generation and interference is eliminated.

Alternatively, a mediator in the form of an insoluble conducting salt NMP-TCNQ, NADP and ICD are co-immobilized at the electrode surface. Only enzyme substrate, isocitrate, is then required for signal generation and this is added in excess to overcome diffusion limitation.

Co-immobilization of ICD, NADP and an insoluble mediator is feasible and practical. It is contemplated that blood interference will be greatly reduced or eliminated

Two-Step Method

Lead inhibition and signal measurement will be separately performed. The bioelectrode is first treated with a blood sample containing lead for a fixed amount of time during which lead ion will inactivate the enzyme. The blood is rinsed off and signal measurement quickly determined Rinsing will remove the blood and reduce blood interference without altering lead ion inhibition. The two-step method will eliminate any blood interference and will remove any potentially interfering species in the blood sample that are electrochemically active.

Alternatively, the blood sample is treated before measurement. Several appropriate methods of treatment are contemplated.

Dilution of Sample

The blood sample will be diluted with a buffer containing a detergent such as SDS to hemolyze the blood, or, treated with a lead complexing agent. Appropriate dilution of the sample will reduce blood interference to a tolerable level and may facilitate the inhibition process.

channel acts as a reference electrode, the bare gold channel acts as a counter electrode, and the colloidal gold/enzyme coated channel acts as a working electrode The three-electrode strip responded to a standard enzyme substrate in a manner comparable to single channel enzyme sensors.

The tri-electrode strip will fit into the tip of a reusable electronic analysis and display unit, making an electrical connection with contacts on the inserted end of the strip. A small volume of sample is placed on the sensing end of the strip and catalytic current determined from a digital readout.

PROPHETIC EXAMPLE 10

The following examples illustrates a contemplated method for determination of lead ion in various materials where detection may be considered important

Detection of Lead Ion in Urine

A 10 μl urine sample is adjusted to a pH of about 8.5, then measured for lead ion as in Example 1.

Detection of Lead Ion in Wastewater

Measurement of lead in wastewater is conducted as in Example 1. Controls will determine if interfering metal ions are present (Sheikh and Townshend, 1974).

REFERENCES

The references listed below are incorporated herein by reference to the extent that they supplement, explain, provide a background for or teach methodology, techniques and/or compositions employed herein.

Albery, W.J., Cass, A.E.G., Mangold, B.P. and Shu, Z.X. *Biosensors & Bioelectronics* 5, 397 (1990).

Almestrand, L., Betti, M., Hua, C., Jagner, D. and Renman, L., *Anal. Chim. Acta* 209, 339–343 (1988).

Baum, P. and Czok, R., Biochem. Z. 332, 121 (1959).

Botre, C., Botre, F., Jommi, G. and Signorini, R., J. Med. Chem. 29, 1814 (1986).

Chemical and Engineering News, page 17, Oct. 14, 1991.

Fair, B.D. and Jamieson, A.M., *J. Colloid Interface Sci.* 77, 525 (1980).

Guilbault, G.G., Brignac, P., Jr., and Zimmer, M., *Anal. Chem.* 40, 190–196 (1968).

Guilbault, G.G., "*Enzymatic Methods of Analysis*", Pergamon Press, 1970.

Gunasingham, H., Delangin, R.R., Fleet, B., Narayanan, B. and Tang, T.F., *Instruments for Science,* Cole-Palmer Instrument Company, Chicago, IL, 1989).

Holleck, G.L., *J. Electrochem. Soc.* 119, 1158 (1972).

Kamata, S. and Onoyama, K., *Anal. Chem.* 63, 1295 (1991). Kratochvil, B., Boyer, S.L., and Hicks, G.P., *Anal. Chem.* 39, 45–51 (1967).

Linde, H.W., *anal. Chem.* 31, 2092 (1959).

Mealor, D., and Townshend, A., *Talanta* 15, 747 (1968).

Morrissey, B.W. and Han, C.C., *J. Colloid Interface Sci.* 65, 423 (1976).

Sheikh, R.A. and Townshend, A., *Talanta* 21, 401–409 (1974).

Smit, M.H. and Cass, A.E.G., *Anal. Chem.* 62, 2429–2436 (1990).

Toren, E.C. and Burger, F.J., *Mikrochimica Acta (wien),* 5389–545 (1968).

Tran-Minh, C. Pandey, P.C. and Kumaran, S., *Biosensors & Bioelectronics* 5, 461 (1990).

What is claimed is:

1. A surface-modified bioelectrode for detecting nanomolar levels of lead or mercury ion consisting essentially of a colloidal gold adsorbed enzyme deposited onto an d in contact with a conducting surface to form a coating thickness of between about 4–12 monolayer a on said conducting surface wherein inhibition of the enzyme detectably affects current generated from redox reactions catalyzed by the enzyme when the surface modified bioelectrode is suitably coupled wit a reference electrode.

2. The biodelectrode of claim **1** wherein the enzyme comprises an oxidase or a dehydrogenase.

3. The bioelectrode of claim **1** wherein the enzyme comprises isocitrate dehydrogenase.

4. The biodelectrode of claim **1** wherein the enzyme comprises alcohol dehydrogenase.

5. The bioelectrode of claim **1** wherein the inhibition by lead or mercury ion is irreversible.

6. The bioelectrode of claim **1** further comprising a mediator.

7. The bioelectrode of claim **6** wherein he mediator comprises N-methylphenazine methosulfate, phenoxazine, ferrocene or N-methylphenazine.

8. The bioelectrode of claim **1** wherein the enzyme/-colloidal gold is deposited on an electrode surface by evaporation.

9. The bioelectrode of claim **1** wherein the enzyme/-colloidal gold is deposited on an electrode surface by electrodeposition.

10. The bioelectrode of claim **1** wherein the conducting surface is glassy carbon.

11. The biosensor of claim **1** further comprising dispersing the enzyme within a membranous or gelatinous film located on the bioelectrode surface.

12. The biosensor of claim **11** wherein the bioelectrode surface is gold, glassy carbon, colloidal gold or platinum.

13. The biosensor of claim **11** wherein a cofactor or mediator is included within the film.

14. A bioelectrode of the detection of lead ion concentrations of a least 10 μg/dl consisting essentially of colloidal gold adsorbed isocitrate dehydrogenase deposited to form a coating thickness of between about 4–12 nonlayers on a glassy carbon surface with AND or NADP.

15. The biodelectrode of claim **14** wherein the colloidal gold is between about 20 and 60 nm diameter.

16. The bioelectrode of claim **14** further comprising a mediator.

17. The bioelectrode of claim **16** wherein the mediator is N-methylphenazine methosulfate.

18. A biosensor comprising a reference electrode and a bioelectrode according to claim **1**, said bioelectrode having a surface near or one which is located an enzyme capable of catalyzing a redox reaction wherein presence of lead or mercury ion concentrations less tan about 10 μg/dl adversely affects a current generated in the presence of a substrate.

19. The biosensor of claim **18** wherein he metal ion is lead.

20. The biosensor of claim **18** wherein the enzyme is isocitrate dehydrogenase.

21. The biosensor of claim **18** wherein he metal ion is mercury.

22. The biosensor of claim **18** wherine he enzyme is alcohol dehydrogenase.

23. An apparatus for detecting metal ion levels in fluids, comprising:

a receptacle having walls for containing a fluid sample;

a biosensor in accordance with claim **18** placed to allow contact with said fluid sample; and

a detector positioned to receive a signal from the biosensor

wherein the signal is obtained from current generated from a redox reaction catalyzed by the enzyme located on the bioelectrode surface.

24. The apparatus of claim **23** wherein the current generated is inversely related to an amount of metal ion present in the fluid sample.

25. The apparatus of claim **23** further comprising a counter electrode.

26. The apparatus of claim **23** further comprising an electronic analysis and display component.

27. The apparatus of claim **26** wherein the electronic analysis and display component is reusable.

28. The apparatus of claim **23** wherein the bioelectrode is disposable.

29. The apparatus of claim **23** wherein the metal ion detected is lead ion.

30. The apparatus of claim **23** wherein the metal ion detected is mercury ion.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,217,594

DATED : June 8, 1993

INVENTOR(S) : Henkens *et al.*

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In column 15, line 15, delete "illustrates" and replace with --illustrate--.

In column 16, line 2, delete "an d" and replace with --and--.

In column 16, line 4, delete "layer a" and replace with --layers--.

In column 16, line 7, change "wit" to --with--.

In column 16, line 13, change "biodelectrode" to --bioelectrode--.

In column 16, line 19, change "he" to --the--.

In column 16, line 38, change the first "of" to --for--.

In column 16, line 42, change "AND" to --NAD--.

In column 16, line 52, change "one" to --on--.

In column 16, line 54, change "tan" to --than--.

In column 16, line 47, change "he" to --the--.

In column 16, line 61, change "he" to --the--.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,217,594

DATED : June 8, 1993

INVENTOR(S) : Henkens et al.

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

In column 16, line 63, delete "wherine he" and replace with --wherein the--.

Signed and Sealed this

Twenty-fifth Day of January, 1994

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks