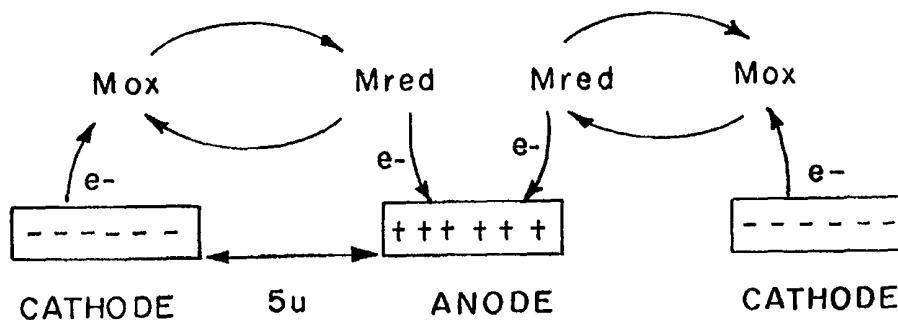




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(54) Title: METHOD AND DEVICE FOR ELECTROCHEMICAL IMMUNOASSAY OF MULTIPLE ANALYTES



(57) Abstract

A method and device for detection and quantification of biologically significant analytes in a liquid sample is described. The method includes contacting a volume of a liquid sample with predetermined amounts of at least a first and second redox reversible species having redox potentials differing by at least 50 millivolts. At least one of the redox reversible species comprises a liquid sample diffusible conjugate of a ligand analog of an analyte in the liquid sample and a redox reversible label. A predetermined amount of at least one specific binding partner for each analyte to be measured is combined with the sample and current flow is measured at first and second anodic and cathodic potentials and correlated with current flows for known concentrations of the respective diffusible redox reversible species. Diagnostic devices and kits, including such devices and the specified specific binding partner(s) and redox reversible species are also described.

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METHOD AND DEVICE FOR ELECTROCHEMICAL
IMMUNOASSAY OF MULTIPLE ANALYTES

5 Field of the Invention

This invention relates to a method and device for detection and quantification of biologically significant analytes in a liquid sample. More particularly the invention is directed to a biosensor and method of using same for electrochemical immunoassays of multiple analyte species in a single liquid sample.

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Background and Summary of the Invention

Therapeutic protocols used today by medical practitioners in treatment of their patient population requires accurate and convenient methods of monitoring patient disease states. Much effort has been directed to research and development of methods for measuring the presence and/or concentration of biologically significant substances indicative of a clinical condition or disease state, particularly in body fluids such as blood, urine or saliva. Such methods have been developed to detect the existence or severity of a wide variety of disease states such as diabetes, metabolic disorders, hormonal disorders, and for monitoring the presence and/or concentration of ethical or illegal drugs. More recently there have been significant advancements in the use of affinity-based electrochemical detection/measurement techniques which rely, at least in part, on the formation of a complex between the chemical species being assayed (the "analyte") and another species to which it will bind specifically (a "specific binding partner"). Such methods typically employ a labeled ligand analog of the target analyte, the ligand analog selected so that it binds competitively with the analyte to the specific binding partner. The ligand analog is labeled so that the extent of binding of the labeled ligand analog with the specific binding partner can be measured and correlated with the presence and/or concentration of the target analyte in the biological sample.

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Numerous labels have been employed in such affinity based sample analysis techniques, including enzyme labeling, radioisotopic labeling, fluorescent labeling, and labeling with chemical species subject to electrochemical oxidation and/or reduction. The use of redox reversible species, sometimes referred to as electron transfer agents or electron mediators as labels for ligand analogs, have proven to

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provide a practical and dependable results in affinity-based electrochemical assays. However, the use of electrochemical techniques in detecting and quantifying concentrations of such redox reversible species (correlating with analyte concentrations) is not without problem. Electrochemical measurements are subject to many influences that affect the accuracy of the measurements, including those relating to variations in the electrode structure itself and/or matrix effects deriving from variability in liquid samples.

The present invention relates to immunosensors based on direct electrochemical measurement of detectable species with microarray electrodes under bipotentiostatic control. An electrochemical label, for example an Os mediator, is covalently attached to a peptide which has amino acid sequence of the binding epitope for the antibody. When indicator/peptide conjugate is bound to antibody, the indicator does not function electrochemically or it is said to be "inhibited". The analyte present in sample will compete with indicator/peptide conjugate for the limited number of binding sites on the antibody. When more analyte is present, more free indicator/peptide conjugate will be left producing higher current at a sensor electrode, *i.e.*, one of the working electrodes where measured events (oxidation or reduction) are taking place. In the opposite case, when less analyte is present, more indicator/peptide conjugate will be bound to antibody resulting less free conjugates and producing lower current levels at the working electrodes. Therefore the current detected at either one of the working electrodes will be a function of analyte concentration.

It is frequently desired to measure more than one analyte species in a liquid sample. Measurement of multiple species in a mixture has been achieved with photometry and fluorescence, via selection of the appropriate wavelengths. Electrochemical measurements of a single species in a complex mixture are routinely made by selecting a potential at which only the desire species is oxidized or reduced (amperometry) or by stepping or varying the potential over a range in which only the desired species changes its electrochemical properties (AC and pulse methods). These methods suffer from disadvantages including lack of sensitivity and lack of specificity, interference by charging and matrix polarization currents (pulse methods) and electrode fouling due to the inability to apply an adequate overpotential. Moreover,

electrochemical measurements are complicated by interference between the multiplicity of electroactive species commonly extant in biological samples.

Electrode structures which generate steady state current via diffusional feedback, including interdigitated array electrodes (IDAs) (Figs. 1 and 2) and parallel plate arrangements with bipotentiostatic control are known. They have been used to measure reversible species based on the steady state current achieved by cycling of the reversible species. A reversible mediator (redox reversible species) is alternately oxidized and reduced on the interdigitated electrode fingers. The steady state current is proportionate to mediator concentration (Fig. 3) and limited by mediator diffusion. A steady state current is achieved within seconds of applying the predetermined anodic (more positive) and cathodic (less positive or negative) potentials (Fig. 6) to the microelectrode array. The slope of a plot of the IDA current vs. mediator concentration is dependent on IDA dimensions, and the slope increases with narrower electrode spacings (Fig. 7).

One embodiment of the present invention provides a method for measuring multiple analyte species in the same sample, and optimally on the same electrode structure, thus improving the accuracy of the relative measurements. This invention also provides an electrochemical biosensor with capacity to provide improved accuracy through the use of self-compensation. Analyte concentration can be measured/calculated from electrometric data obtained on the same liquid sample with the same electrode structure (the working electrodes), thereby minimizing perturbations due to variability in sample or electrode structure.

The various embodiments of this invention utilize the principle of diffusional recycling, where a diffusible redox reversible species is alternately oxidized and reduced at nearby electrodes, thereby generating a measurable current. As alternate oxidation and reduction is required for measurement, only electroactive species which are electrochemically reversible are measured thereby eliminating, or at least reducing, the impact or interference from non-reversible electroactive species in the sample. Redox reversible species having different oxidation potentials can be independently measured in a mixture by selecting and bipotentiostatically controlling the oxidizing and reducing potentials for neighboring electrode pairs so that only the species of interest is oxidized at the anode (the electrode with the more positive

potential) and reduced at the cathode (the electrode with the less positive or negative potential). When the working electrodes (the anode/cathode arrays) are dimensioned to allow diffusional recycling of the redox-reversible-species at the selected oxidizing and reducing potentials appropriate for that species, a steady state current at the
5 working electrodes where the measurable oxidative and reductive events are taking place, is quickly established through the sample and the electrode structure. The magnitude of the current is proportional to the concentration of the diffusible redox reversible species in the sample. When two or more redox reversible species are utilized, they are selected to have redox potentials differing by at least 50 millivolts,
10 most preferably at least 200 millivolts, to minimize interference between one species and the other in measurements of the respective steady state currents.

Any electrode structure which allows for diffusional recycling to achieve steady state current in response to application of pre-selected species-specific anodic and cathodic potentials can be utilized in carrying out the invention. Suitable
15 electrode structures include interdigitated array microelectrodes and parallel plate electrodes separated by distances within the diffusion distance of the respective redox reversible species. The electrode structures typically include a reference electrode (*e.g.*, Ag/AgCl), at least two working electrodes (one at positive potential and another at a less positive or negative potential relative to the reference electrode), and optionally an
20 auxiliary electrode for current control. In use, a programmable bipotentiostat is placed in electrical communication with the electrode structure for applying the respective anodic and cathodic potentials specific for each of the respective redox reversible species utilized in the method/biosensor. Several novel osmium complexes have been developed for use as labels for preparing ligand analog conjugates having potential
25 differences sufficient to allow the use of two osmium complexes (as opposed to an osmium complex and a ferrocene or other redox reversible label) in this invention.

Accordingly, one embodiment of the invention provides a device for detecting or quantifying one or more analytes in a liquid sample. The device comprises at least two redox reversible species having respective redox potentials differing by at
30 least 50 millivolts, and an electrode structure for contact with the liquid sample. In one embodiment the device further comprises a chamber for containing the liquid sample, optionally dimensioned for capillary fill. The electrode structure includes a

reference electrode and an anode and a cathode (working electrodes) dimensioned to allow diffusional recycling of the redox reversible species in the sample when a redox-reversible-species-dependent cathodic potential is applied to one working electrode and a redox-reversible-species-dependent anodic potential is applied to a second
5 electrode to enable and sustain a measurable current through the sample. The device also includes conductors communicating with the respective electrodes for applying potentials and for carrying current conducted between the sample and the respective electrodes.

The device in accordance with this invention is typically utilized in
10 combination with a meter which includes a power source, for example a battery, a microprocessor, a register for storing measured current values, and a display for reporting calculated analyte concentrations based on measured current values. The construction and configuration of such meters are well known in the art. Meters for use in accordance with the present device further comprise a bipotentiostat under
15 control of the microprocessor or separately programmable to apply predetermined potentials to the device component microelectrode arrays during liquid sample analysis. Improvements in meter construction and design for biosensor systems are described in U.S. Patent Nos. 4,999,632; 5,243,516; 5,366,609; 5,352,351; 5,405,511; and 5,48,271, the disclosures of which are hereby incorporated by reference.

20 In another embodiment of the invention there is provided a method for measuring the concentration of one or more analytes in a liquid sample. The method includes contacting a portion of the sample with pre-determined amounts of at least a first and second redox reversible species having a redox potential differing by at least 50 millivolts from that of each other species. Each respective species comprises a
25 liquid sample diffusible conjugate of a ligand analog of an analyte in the liquid sample and a redox reversible label. The liquid sample is also contacted with a predetermined amount of at least one specific binding partner for each analyte to be measured. The diffusible conjugate is selected so that it is capable of competitive binding with the specific binding partner for said analyte.

30 The concentration of diffusible redox-reversible-species in the liquid sample is then determined electrochemically. The sample is contacted with an electrode structure, including a reference electrode and at least first and second

working electrodes dimensioned to allow diffusional recycling of at least one of the diffusible redox-reversible-species in the sample, when a predetermined redox-reversible-species-dependent cathodic potential is applied to one working electrode and a predetermined redox-reversible-species-dependent anodic potential is applied to the second working electrode. Typically, a first cathodic potential is applied to the first working electrode and a first anodic potential is applied to the second working electrode to establish current flow through the sample due to diffusional recycling of the first redox-reversible-species without significant interference from the second redox-reversible-species. Current flow through one or more of the electrodes at the first anodic and cathodic potentials is measured. Similarly current flow responsive to application of second cathodic and anodic potentials to electrodes in contact with the sample is measured and correlated with measured current flows for known concentrations of the respective redox-reversible-species, said concentrations being proportionate to the respective analyte concentrations at a predetermined redox-reversible species-dependent potential (anodic or cathodic). Alternatively, the potential of one of the working electrodes can be held constant and current flow is monitored as the potential of the other working electrode is varied and swept through the other redox-reversible species-dependent potential.

The reagent components for the invention, including the redox reversible species and the specific binding partners, can be provided in the form of a test kit for measuring the targeted analyte(s) in a liquid sample, either as separate reagents or, more preferably, combined as a multi-reagent composition, *e.g.* combined redox reversible species, combined specific binding partners, or combined redox reversible species and specific binding partners. The kit optionally, but preferably, includes an electrode structure dimensioned to allow diffusional redox recycling of diffusible redox reversible species in the liquid sample. The electrode structure includes conductors for connecting the structure bipotentiostat programmed to apply redox-reversible-species-dependent-anodic and cathodic potentials to the electrode structure and to sense and measure current flow, typically at one or both of the working electrodes, responsive to such applied potentials.

Also described herein is the preparation and use of electrochemically detectable osmium complexes and covalent conjugates of said complexes having

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oxidation potentials differing sufficiently to enable their use together in the respective method and device embodiments of the invention. Osmium labeled ligand analogs capable of binding to a specific binding partner of a biologically significant analyte are prepared. One group of electrochemically detectable conjugates comprise a

5 *bis*(bipyridyl) imidazolyl chloroosmium complex characterized by fast mediation kinetics and low redox potential (+150mV vs. Ag/AgCl). Another group of osmium complex labeled, electrochemically detectable conjugates include *tris*(bipyridyl) osmium complexes, which, like the *bis*(bipyridyl) imidazolyl chloroosmium complexes are characterized by fast mediation kinetics, but the *tris*(bipyridyl) complexes have a

10 redox potential sufficiently different from the *bis*(pyridyl) imidazolyl chloroosmium complexes to allow their use together in the various embodiments of this invention to enable use of microelectrode arrays for measuring more than one analyte in a single liquid sample by concentration dependent currents amplified by diffusional redox recycling.

15 In one preferred embodiment of the invention at least one osmium complex conjugates is used in combination with another conjugated redox-reversible-species for the measurement of both glycosylated hemoglobin and hemoglobin in a lysed blood sample. One redox-reversible-species preferably comprises an osmium complex covalently linked to a ligand analog of either hemoglobin or glycosylated

20 hemoglobin, and the second redox-reversible-species comprises a second redox reversible label covalently bound to a ligand analog of the other of the two target analytes. The method enables measurement of the concentration of both the glycosylated hemoglobin (HbA_{1c}) and the concentration of either total hemoglobin or that of unglycosylated hemoglobin (HbA₀) thereby enabling calculation of the results as

25 a ratio of the two measurements (% HbA_{1c}). It is advantageous to assay both HbA_{1c} and total hemoglobin (or HbA₀) using the same principle in a single sample.

Brief Description of the Drawings

Fig. 1 is an enlarged plan view of an interdigitated array electrode for

30 reversible mediator measurement in accordance with the present invention.

Fig. 2 is a partial cross-sectional view of the electrode of Fig. 1 illustrating the conditions of steady state current limited by diffusion of reversible

mediator (M) which is alternately oxidized and reduced on the interdigitated electrode fingers.

Fig. 3 is a graphic presentation of dose response currents for a *bis*-(bipyridyl) imidazolyl chloroosmonium mediator in a peptide conjugate of that mediator.

Fig. 4 is a graphic illustration of current flow vs. concentration of glycosylated hemoglobin (HbA1c) in blood samples using an osmium conjugate and enzyme amplified DC amperometry.

Fig. 5 is a graphic illustration of the inhibition of current flow due to free conjugate as a function of antibody concentration (C_n) as measured using enzyme amplified DC amperometry [$C_1 > C_2 > C_3$].

Fig. 6 is a graphic illustration of current flow vs. time using an interdigitated array electrode.

Fig. 7 is a graphic illustration of the effect of the dimensions of the interdigitated array electrode structure on current flow as a function of concentration of an osmium conjugate (Os-DSG-A1c).

Fig. 8 is a graphic illustration of current flow as a function of applied potential for a liquid sample containing equimolar ($50\mu\text{M}$) of a *bis*-(bipyridyl) imidazolyl chloroosmium complex and a *tris*(bipyridyl) osmium complex.

Fig. 9 is a graphic presentation of current flow vs. concentration of a ferrocene-biotin conjugate in the presence of varying amounts of an osmium complex conjugate on interdigitated array electrodes with bipotentiostatic control.

Fig. 10 is a graphic illustration of the effect of concentration of an unlabeled conjugate (BSA-A1c) on current flow in a solution containing osmium labeled conjugate (osmium-DSG-A1c) in the presence of three separate A1c-recognizing antibody compositions.

Fig. 11 illustrates the structure of a *tris*(bipyridyl) osmium labeled conjugate for use in accordance with this invention.

Figs. 12-14 are similar and each depict the chemical structure of a *bis*(bipyridyl) imidazolyl chloroosmium labeled peptide conjugate for use in accordance with this invention.

Detailed Description of the Invention

One aspect of the invention is a method for measuring the concentration of one or more analytes in a liquid sample. The method enables two or more independent amperometric measurements of the sample on a single electrode structure.

5 The method comprises

contacting a volume of said liquid sample with

1) predetermined amounts of at least a first and second redox reversible species, each respective species having a redox potential differing by at least 50 millivolts from that of each other species, at least one species comprising a liquid sample diffusible conjugate of a ligand analog of an analyte in the liquid sample and a redox reversible label, said conjugate capable of competitive binding with a specific binding partner for said analyte, and

2) a predetermined amount of at least one specific binding partner for each analyte to be measured; and

15 electrochemically determining the concentration of each of said diffusible redox-reversible species in the liquid sample by

contacting said sample with an electrode structure including a reference electrode and at least first and second working electrodes dimensioned to allow diffusional recycling of the diffusible redox reversible species in the sample when a predetermine redox-reversible-species-dependent cathodic potential is applied to one working electrode and a predetermined redox-reversible-species-dependent anodic potential is applied to a second working electrode, said diffusional recycling of said species being sufficient to sustain a measurable current through said sample,

25 applying a first cathodic potential to the first working electrode and a first anodic potential to the second working electrode, said first cathodic and anodic potentials corresponding to those respective potentials necessary to establish current flow through the sample due to diffusional recycling of the first redox reversible species without significant interference from said second redox reversible species,

30 measuring current flow at said first anodic and cathodic potentials,

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applying a second cathodic potential to said first or second working electrode and a second anodic potential to the other working electrode, said second cathodic and anodic potential corresponding to those respective potentials necessary to establish current flow through the sample due to diffusional recycling of the second redox-reversible-species without significant interference from the first redox reversible species,

measuring current flow at said second anodic and cathodic potentials, and

correlating the respective measured current flows to that for known concentrations of the respective diffusible redox reversible species.

The method of the invention has very broad applicability but in particular may be used to assay: drugs, hormones, including peptide hormones (*e.g.*, thyroid stimulating hormone (TSH), luteinizing hormone (LH), follicle stimulating hormone (FSH), insulin and prolactin) or non-peptide hormones (*e.g.*, steroid hormones such as cortisol, estradiol, progesterone and testosterone, or thyroid hormones such as thyroxine (T4) and triiodothyronine), proteins (*e.g.*, human chorionic gonadotropin (hCG), carcino-embryonic antigen (CEA) and alphafetoprotein (AFP)), drugs (*e.g.*, digoxin), sugars, toxins or vitamins.

The method can be performed on liquid samples comprising biological fluids such as saliva, urine, or blood, or the liquid sample can be derived from environmental sources. The liquid samples can be analyzed "as is," or they can be diluted, buffered or otherwise processed to optimize detection of the targeted analyte(s). Thus, for example, blood samples can be lysed and/or otherwise denatured to solubilize cellular components.

The method can be performed using widely variant sampling handling techniques. Thus, the sample can be premixed with either or both of the specific binding partner for the targeted analytes and the redox reversible species prior to contacting the sample with the electrode structure, or the liquid sample, either neat or pre-processed, can be delivered to a vessel containing predetermined amounts of the redox reversible species and the specific binding partner for subsequent or simultaneous contact with the electrode structure. The order of introduction of the components into the sample is not critical; however, in one embodiment of the

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invention the predetermined amounts of the specific binding partners are first added to the sample, and thereafter, there is added the predetermined amounts of the redox reversible species. It is also possible to combine the predetermined amounts of the specific binding partners with the redox reversible species to form the respective complexes prior to combining those components with the liquid sample. In that latter case the redox reversible species will be displaced from its respective specific binding partner by the corresponding analyte to provide a concentration of the redox reversible species proportionate to the concentration of analyte in the liquid sample. The reagents, that is, the predetermined amounts of the specific binding partner of each analyte and the predetermined amounts of the corresponding redox reversible species can, for example, be deposited in a vessel for receiving a predetermined volume of the liquid sample. The liquid sample is added to the vessel, and thereafter, or simultaneously, the liquid sample is contacted with the electrode structure.

The electrode structure includes a reference electrode and at least first and second working electrodes dimensioned to allow diffusional recycling of the diffusible redox reversible species in the sample when predetermined redox-reversible-species-dependent-cathodic and anodic potential is applied to the working electrodes. The term "working electrode" as used herein refers to an electrode where measured events (*i.e.* oxidation and/or reduction) take place and resultant current flow can be measured as an indicator of analyte concentration. "Anodic potential" refers to the more positive potential (applied to the anode) and "cathodic potential" refers to the less positive or negative potential applied to the cathode (vs. a reference electrode). Electrodes dimensioned to allow diffusional recycling are well known in the art and are typically in the form of arrays of microdiscs, microholes, or microbands. In one embodiment the electrodes are in the form of an interdigitated arrangement of microband electrodes with micron or submicron spacing. Short average diffusional length and a large number of electrodes are desirable for effective current amplification by recycling of reversible redox species. The microelectrode arrays can be fabricated, for example, as pairs of interdigitated thin film metal electrodes in micron and submicron geometry arranged on an insulator substrate, for example, oxidized silicon. Each of the electrode fingers (Fig. 1) are spaced from its neighboring finger in the nanometer to low micrometer (1-10 microns) range. Microelectrode arrays can be

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fabricated using photolithography, electron beam lithography, and so-called lift-off technique. Thus, an interdigitated electrode array (IDA) can be deposited on glass, silicon or polyamide utilizing the following general procedure:

1. Grow thermal oxide layer on silicon substrate;
- 5 2. Sputter 400Å chromium seed layer, 2000 Å gold;
3. Spin-coat and soft-bake photo resist;
4. Expose and develop photo resist with IDA pattern;
5. Pattern gold and chromium with ion beam milling;
6. Strip photo resist; and
- 10 7. Cut electrodes into chips by first coating with a protective layer, cutting into strips, stripping the protective layer, and cleaning electrode surfaces in oxygen plasma.

The electrode structure can be formed on an inner surface of a chamber for receiving the liquid sample, *e.g.*, a cuvette, a capillary fill chamber, or other sample receiving vessel wherein the electrode structure can be contacted with the liquid sample. Alternatively, the electrode structure can form part of a probe for dipping into the liquid sample after the sample has been contacted with the predetermined amounts of the redox reversible species and the specific binding partners. The electrode structure is in contact with conductors that enable application of the respective cathodic and anodic potentials for carrying out the present method. The anodic and cathodic potentials are applied relative to a reference electrode component of the electrode structure using a bipotentiostat. The electrode structure can optionally include an auxiliary electrode for current control. The bipotentiostat is utilized to apply a first cathodic potential to a first working electrode and a first anodic potential to a second working electrode, the first cathodic and anodic potentials corresponding to those respective potentials necessary to establish current flow through the sample due to diffusional recycling of the first redox reversible species. Optionally the potential on one working electrode can be set at a first diffusible species dependent, anodic potential and current flow is measured as the potential of the other working electrode is swept through a potential corresponding to the predetermined diffusible species dependent cathodic potential (or *vice versa*).

The cathodic and anodic potentials appropriate for each reversible redox species can be readily determined by empirical measurement. The multiple redox reversible species used in performance of the method of this invention are selected to have redox potentials differing by at least 50 millivolts, more preferably at least 100 millivolts, more preferably at least 200 millivolts, from that of each other redox reversible species utilized in the method. The difference in redox potentials of the redox reversible species being used allow each species to be detected without significant interference from the second or any other redox reversible species in the liquid sample. A steady state current flow is rapidly established at each of the working electrodes following application of the anodic and cathodic potentials. Current flow can be measured at either or both working electrodes, and it is proportionate to the concentration of the recycling redox reversible species.

Second cathodic and anodic potentials are applied to the working electrodes wherein said second potentials correspond to those respective potentials necessary to establish current flow through the sample due to diffusional recycling of the second redox reversible species without significant interference from the first redox reversible species, and the resulting steady state current flow is measured. This step is repeated for each redox reversible species utilized in the method. The measured current flows are then correlated to known concentrations of the respective diffusible redox reversible species. Those concentrations are proportionate to the respective analyte concentrations.

The method steps can be conducted using a programed bipotentiostat to control potentials on the electrode structure in contact with the sample. The bipotentiostat can be included either in a desktop or hand-held meter further including means for reading values for steady state current, storing said values, and calculating analyte concentrations using a microprocessor programmed for making such calculations.

The redox reversible species utilized in the method comprise a liquid-sample-diffusible conjugate of a ligand analog of an analyte in the liquid sample and a redox reversible label. The term "ligand analog" as used in defining the present invention refers to a chemical species capable of complexing with the same specific binding partner as the analyte being measured and can include the analyte itself,

provided that the molecular weight of the conjugate is less than about 50,000, more preferably less than about 10,000 Daltons. Most preferably the molecular weight of the conjugate of the ligand analog and the redox reversible label is between about 500 and about 5,000 Daltons. Low molecular weight redox reversible species are most desirable in view of the diffusion-based electrochemical detection technique utilized in carrying out the present method.

The term "redox reversible label" as used herein refers to a chemical species capable of reversible oxidation and reduction in a liquid sample. It can be in the form of an organic moiety, for example, a chemical group comprising a nitrosoaniline, a catechol, hydroquinone, or an aminophenol group. Alternatively, the redox reversible label can be an inorganic or organometallic species capable of undergoing reversible oxidation and reduction in a liquid sample. Such species may be, for example, complete molecules, portions of molecules, atoms, ions, or more particularly, ion complexes. Redox reversible labels are well-known in the art and include ligand complexes of transition metal ions, for example iron (ferrocene and ferrocene derivatives), ruthenium and osmium.

The relative amounts of the first and second redox reversible species and the respective specific binding partners for the targeted analytes to be measured in the method can be determined empirically. They are dependent on the concentration ranges of the targeted analyte, and the binding stoichiometry of the specific binding partner, the binding constant, the analyte and the corresponding redox reversible species. The amounts of each reagent appropriate for each analyte being measured can be determined by empirical methods.

The redox reversible species typically comprises a conjugate of a ligand analog of an analyte in a liquid sample and a redox reversible label. The conjugate is prepared by linking the ligand analog to the label either covalently through bifunctional linking agents or by combination of covalent linkages and art-recognized specific binding entities (for example, biotin-avidin).

In one embodiment of the invention the specific binding partner for each analyte is an antibody and the ligand analog is selected so that it binds competitively with the analyte to the antibody. There are, however, other examples of ligand-specific binding partner interactions that can be utilized in developing

applications of the present method. Examples of ligands and specific binding partners for said ligands are listed below.

	<u>Ligand</u>	<u>Specific Binding Partner</u>
5	Antigen (<i>e.g.</i> , a drug substance)	Specific antibody
	Antibody	Antigen
	Hormone	Hormone receptor
	Hormone receptor	Hormone
	Polynucleotide	Complementary polynucleotide strand
10	Avidin	Biotin
	Biotin	Avidin
	Protein A	Immunoglobulin
	Immunoglobulin	Protein A
	Enzyme	Enzyme cofactor (substrate)
15	Enzyme cofactor (substrate)	Enzyme
	Lectins	Specific carbohydrate
	Specific carbohydrate of lectins	Lectins

20

The term "antibody" refers to (a) any of the various classes or subclasses of immunoglobulin, *e.g.*, IgG, IgM, derived from any of the animals conventionally used, *e.g.*, sheep, rabbits, goats or mice; (b) monoclonal antibodies; (c) intact molecules or "fragments" of antibodies, monoclonal or polyclonal, the fragments being those which contain the binding region of the antibody, *i.e.*, fragments devoid of the Fc portion (*e.g.*, Fab, Fab¹, F(ab')₂) or the so-called "half-molecule" fragments obtained by reductive cleavage of the disulfide bonds connecting the heavy chain components in the intact antibody. The preparation of such antibodies are well-known in the art.

30

The term "antigen" used in describing and defining the present invention includes both permanently antigenic species (for example, proteins, peptides, bacteria, bacteria fragments, cells, cell fragments, drug substances, and viruses) and haptans which may be rendered antigenic under suitable conditions.

35

In one embodiment of the invention there is provided a method for measuring two proteinaceous analytes in a liquid sample wherein the ligand analog component of the first redox reversible species is a peptide comprising an epitope of a first analyte and the ligand analog component of a second redox reversible species is a peptide comprising an epitope of a second analyte. One specific binding partner

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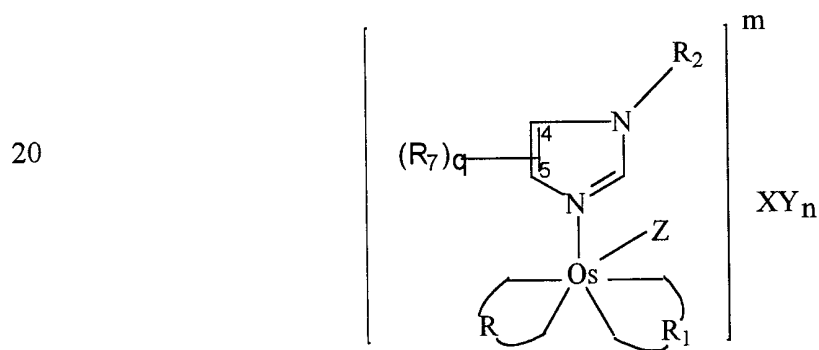
utilized in the method is an antibody recognizing the epitope of the first analyte, and the other specific binding partner is an antibody recognizing the epitope of the second analyte. In another application of the present method two independent measurements are performed on a single analyte in a liquid sample. In that embodiment the respective

5 ligand analog component of the first and second redox reversible species are different ligand analogs of the targeted analyte. Where the targeted analyte is a proteinaceous compound, the ligand analog component of the first redox reversible species is a peptide comprising a first epitope of the analyte, and the ligand analog of the second redox reversible species is a peptide comprising a second epitope of the analyte, and

10 the specific binding partners are first and second antibodies, each recognizing respective first and second analyte epitopes.

In one preferred embodiment of the invention, at least one of the redox reversible labels is an osmium complex. In another embodiment, both redox reversible labels are osmium complexes, each having an oxidizing potential difference of at least

15 50, most preferably at least 200 millivolts. Illustrative of the redox reversible osmium complexes for use in this invention are complexes of the formula



25 wherein

R and R₁ are the same or different and are 2,2'-bipyridyl, 4,4'-disubstituted-2,2'-bipyridyl, 5-5'-disubstituted-2,2'-bipyridyl, 1,10-phenanthrolyl, 4,7-disubstituted-1,10-phenanthrolyl, or 5,6-disubstituted-1,10-phenanthrolyl, wherein each substituent is a methyl, ethyl, or phenyl group,

30 R and R₁ are coordinated to Os through their nitrogen atoms;

q is 1 or 0;

R₇ is B-(L)_k-Q(CH₂)_i -;

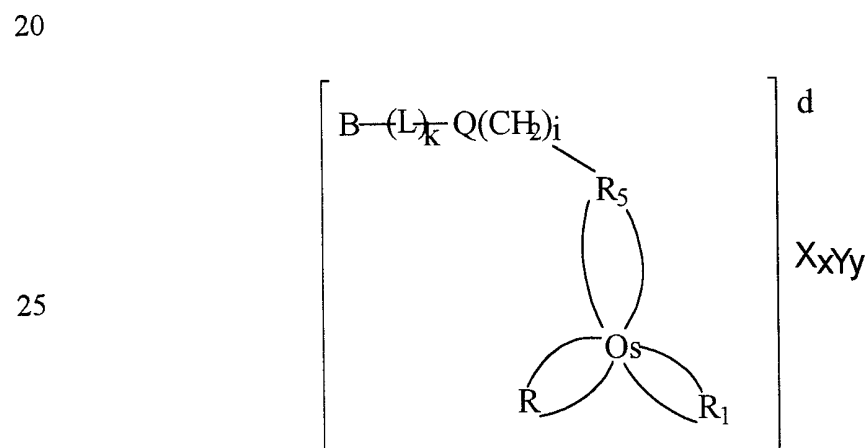
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R_2 is hydrogen, methyl, or ethyl when q is 1, and R_2 is $B-(L)_k-Q(CH_2)_i-$
 when q is 0; wherein in the group $B-(L)_k-Q(CH_2)_i-$
 Q is O, S, or NR_4 wherein R_4 is hydrogen, methyl or ethyl;
 $-L-$ is a divalent linker;
 5 k is 1 or 0;
 i is 1, 2, 3, 4, 5 or 6; and
 B is hydrogen or a group comprising a ligand capable of binding
 to a specific binding partner;

Z is chloro or bromo;
 10 m is +1 or +2;
 X is a mono- or divalent anion, *e.g.*, chloride, bromide, iodide, fluoride,
 tetrafluoroborate, perchlorate, nitrate, sulfate, carbonate, or sulfite;

Y is monovalent anion, *e.g.*, chloride, bromide, iodide, fluoride,
 tetrafluoroborate, perchlorate or nitrate; and
 15 n is 1 or zero,
 provided that when X is a divalent anion, n is zero,
 and when m is 1, n is zero and X is not a divalent anion.

Another redox reversible osmium complex for use in the present
 method is a compound of the formula



30 wherein

R and R_1 are the same or different and are 2,2'-bipyridyl, 4,4'-
 disubstituted-2,2'-bipyridyl, 5-5'-disubstituted,-2,2'-bipyridyl, 1,10-phenanthrolyl,

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4,7-disubstituted-1,10-phenanthrolinyl, or 5,6-disubstituted-1,10-phenanthrolinyl, wherein each substituent is a methyl, ethyl, or phenyl group,

R_5 is 4-substituted-2,2'-bipyridyl or 4,4'-disubstituted-2,2'-bipyridyl wherein the substituent is the group $B-(L)_k-Q(CH_2)_i-$ and the 4'-substituent is a methyl, ethyl or phenyl group;

R , R_1 and R_5 are coordinated to Os through their nitrogen atoms;

Q is O, S, or NR_4 wherein R_4 is hydrogen, methyl or ethyl;

-L- is a divalent linker;

k is 1 or 0;

10 i is 1, 2, 3, 4, 5 or 6;

B is hydrogen or a group comprising a ligand capable of binding to a specific binding partner;

d is +2 or +3;

15 X and Y are anions selected from monovalent anions, *e.g.*, chloride, bromide, iodide, fluoride, tetrafluoroborate, perchlorate, and nitrate and divalent anions, *e.g.*, sulfate, carbonate or sulfite wherein x and y are independently 0, 1, 2, or 3 so that the net charge of X_xY_y is -2 or -3.

Redox reversible conjugate species of each of those formulas are prepared from the corresponding compounds wherein K is 0 and B is hydrogen by reacting such compounds with either a heterofunctional crosslinker of the formula $S-L'-T$ wherein L' is a divalent linker and S and T are different electrophilic groups capable of reacting with a nucleophilic group to form a covalent bond, or with a homofunctional crosslinker of the formula $S-L'-L'-T$ wherein L' is a divalent linker and S and T are the same electrophilic groups capable of reacting with a nucleophilic group to form a covalent bond. The resulting products are then reacted with ligand analogs using classical coupling reaction conditions to product the conjugate species. The oxidizing potentials of the respective *bis*(bipyridyl) and *tris*(bipyridyl) osmium complexes defined above is such that the respective complexes can be used as reversible redox labels for the respective redox reversible species in performance of the method. Fig. 8 illustrates a cyclic voltammogram for a liquid sample containing equimolar (50 μ M) amounts of a *bis*(bipyridyl) imidazolyl chloroosmium complex and a *tris*(bipyridyl) osmium complex.

In another embodiment of the invention there is provided a device for detecting or quantifying one or more analytes in a liquid sample. The device comprises at least two redox reversible species, each capable of diffusion in said liquid sample at least in the presence of a respective predetermined analyte, said redox reversible species having respective redox potentials differing by at least 50 millivolts, an electrode structure for contact with the liquid sample in said chamber, said electrode structure including a reference electrode and working electrodes dimensioned to allow diffusional recycling of a diffusible redox reversible species in a liquid sample in contact with the electrode structure when a predetermined redox-reversible-species-dependent cathodic potential is applied to one working electrode and a predetermined redox-reversible-species-dependent anodic potential is applied to a second working electrode, said diffusional recycling of said species being sufficient to sustain measurable current through each working electrode, and conductors communicating with the respective electrodes for applying said anodic potential and said cathodic potential and for carrying the current conducted by the electrodes.

The device can be constructed using procedures and techniques that have been previously described in the art for construction of biosensors employing electrometric detection techniques. Thus, for example, the device can include a chamber that has a receiving port, and the chamber is dimensioned so that it fills by capillary flow when the liquid sample is contacted with the sample receiving port. The electrode structure can be formed on a plate that defines a wall of the chamber so that the electrode structure will contact a liquid sample in the chamber. Thus, for example, the device can be constructed using the general procedures and designs described in U.S. Patent No. 5,141,868, the disclosure of which is expressly incorporated herein by reference. The features of the present invention can also be incorporated into other electrochemical biosensors or test strips, such as those disclosed in U.S. Patent Nos. 5,120,420; 5,437,999; 5,192,415; 5,264,103; and 5,575,895, the disclosures of which U.S. Patents are expressly incorporated herein by reference. The device can be constructed to include the predetermined amounts of the redox reversible species and the specific binding partners. For example, a mixture of such reagents can be coated onto a wall of the sample chamber in said device during device construction, so that

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the liquid sample is contacted with the reagent mixture as it is delivered into the chamber for containing the sample. In one embodiment the device is constructed for quantifying a first analyte and a second analyte in liquid sample. The device comprises two redox reversible species, a first redox reversible species comprising a conjugate of a ligand analog of the first analyte and a second redox reversible species comprising a conjugate of a ligand analog of the second analyte, and a specific binding partner for each analyte so that each of said analyte analog conjugates are capable of binding competitively with its respective analyte to a specific binding partner.

In one device embodiment of this invention the device further comprises a bipotentiostat in electrical communication with the conductors for applying a redox-reversible-species-dependent-cathodic potential to one working electrode and a redox-reversible-species-dependent-anodic potential to a second working electrode. The bipotentiostat can be programmed to apply a sequence of potentials to the respective working electrodes. More particularly, the bipotentiostat can be programmed to apply first cathodic potential to a first working electrode and a first anodic potential to a second working electrode, said first anodic and cathodic potentials corresponding to those potentials necessary to establish current flow to the sample due to diffusional recycling of the first redox reversible species. The bipotentiostat is also programmed to apply a second cathodic potential to said first working electrode and a second potential to the second anodic electrode, said second cathodic and anodic potentials corresponding to those potentials necessary to establish current flow through the sample due to diffusional recycling of the second redox reversible species. In an alternate embodiment the device includes first and second redox reversible species, and at least first and second electrode structures for contact with the liquid sample in the chamber, each of said electrode structures comprising a microarray of working electrodes, and means for switching the bipotentiostat between the first and second electrode structures. In preferred device embodiments there is provided means for measuring current flow through the sample at each of the first and second potentials and preferably storing values for said current flows in a register coupled to a microprocessor programmed to calculate analyte concentrations based on said values.

In still another embodiment of the present invention there is provided a kit for measuring the concentration of one or more analytes in liquid sample. The kit comprises

at least two redox reversible species for contact with the liquid sample,
5 each capable of diffusion in the liquid sample at least in the presence of a predetermined analyte, at least one of such species being a conjugate of a ligand analog of an analyte and a redox reversible label, said redox reversible species having respective redox potentials differing by at least 50 millivolts;

a specific binding partner for each analyte;
10 an electrode structure for contact with the liquid sample, said electrode structure including a reference electrode and working electrodes dimensioned to allow diffusional recycling of diffusible redox reversible species in the sample when a predetermined redox-reversible-species-dependent-cathodic potential is applied to one working electrode and a predetermined redox-reversible-species-dependent-anodic
15 potential is applied to the second working electrode, said diffusional recycling of said species means sufficient to sustain a measurable current through the sample; and conductors communicating with the respective electrodes for applying said anodic potential and said cathodic potential and for carrying the current conducted by the electrodes.

20 In one embodiment, the redox reversible species are mixed as a novel composition for contact with the liquid sample. In another embodiment each of the redox reversible species and the specific binding partner for each analyte is mixed as a novel composition for contact with the liquid sample. Preferably, the redox reversible label of at least one of the redox reversible species comprises an osmium complex.

25

Preparation of Os Mediator Labels

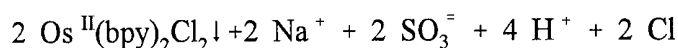
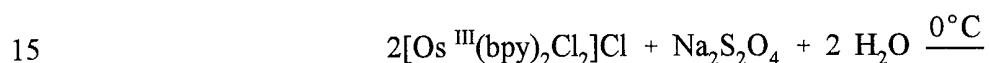
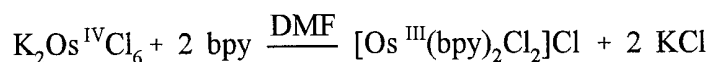
The Os mediator *bis*(bipyridyl) imidazolyl chloroosmium has been shown to be an excellent electron mediator for many oxido-reductase enzymes (U.S. Patent No. 5,589,326). It has fast mediation kinetics (about 500 times faster than
30 ferricyanide with glucose oxidase) and a relatively low redox potential (+150 mV vs. Ag/AgCl). It has also very fast electron transfer rate at electrode surface. More importantly, the organic ligands on Os mediator can be functionalized so that it can be

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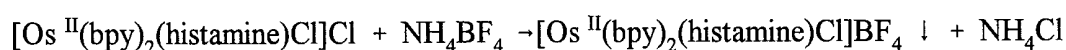
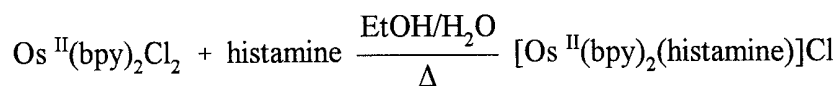
covalently linked to other molecules without detrimental effects on redox properties of the Os center. These unique properties of Os mediator make it an ideal electrochemical indicator for sensors based on immunoaffinity.

Os mediators with these new ligands were synthesized using the same procedure used for Os free mediator. Their synthesis consists of two major process
5 steps as outlined below. Details of these processing steps are described below.

The first process step involves the synthesis of Os intermediate, *cis*-bis(2,2'-bipyridyl) dichloroosmium(II), from commercially available osmium salt using the following scheme. The intermediate product is isolated through recrystallization in
10 an ice bath.



20 The second process step involves the reaction between Os intermediate and histamine or 4-imidazoleacetic acid (or a substituted bipyridine for preparation of the *tris*(bipyridyl) complexes) to produce Os mediators with the appropriate "handle". The desired product is then precipitated out from solution by addition of ammonium tetrafluoroborate.



25

These Os mediators can also be easily converted to oxidized form, *i.e.* Os(III) using nitrosonium tetrafluoroborate. However, this is unnecessary since the Os revert back to reduced form anyway at alkaline conditions during conjugation reactions. And it does not require oxidized form of Os(III) for the detection on the
5 biosensor.

A. Simple Mixed Mediator Measurement

1. Interdigitated array microelectrodes (IDA) are produced through photolithographic means by art-recognized methods, (See W 97/34140; EP
10 0299,780; D.G. Sanderson and LB. Anderson, *Analytical Chemistry*, 57 (1985), 2388; Koichi Aoki et al., *J. Electroanalytical Chemistry*, 256 91988) 269; and D. Niwa et la., *J. Electroanalytical Chemistry*, 167 (1989) 291. Other means which are standard in lithographic processing may also be used to produce the desired patterns of a conductor on insulator substrate.

15 2. Reversible mediators are selected from those described herein and those described references (U.S. Patent Nos.4,945,045 and 5,589,325, the disclosures of which are incorporated herein by reference). Preferably two different mediators are selected with potentials which differ by at least 100 mV, more preferably at least 200 mV. Examples of suitable mediators include the Os(bipy)ImCl described
20 herein and in U.S. Patent No. 5,589,326, the disclosure of which is incorporated herein by reference, and ferrocene, described in U.S. Patent No. 4,945,045 and EP 0142301, the disclosures of which are incorporated herein by reference. Mixtures of these mediators are made in aqueous solution, for example phosphate-buffered saline (PBS). Concentrations between about 1 uM and 1000 uM may conveniently be measured.

25 3. The IDA is connected to a bipotentiostat, an instrument capable of controlling the potential of two separate electrodes. Also provided is a reference electrode. This non-polarizable electrode serves as the reference for the two applied potentials and may also serve as the counter electrode. Any non-polarizable electrode may be used, for example Ag/AgCl, such as may be obtained from ABI/Abtech. An
30 auxiliary electrode can also be used for controlling current flow through the working electrodes. The mixtures are placed on the IDA electrode and the reference electrode

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also contacted with the mixture, or the IDA along with the reference electrode may be dipped into the mixture.

4. To measure Mediator 1 (Os(bipy)₂ImCl)

A cathodic potential is applied to one set of fingers of the IDA which is capable of
5 reducing mediator 1 (ca -50 mV vs. Ag/AgCl). An anodic potential is applied to the
other set of fingers of the IDA which is capable of oxidizing mediator 1 but not
mediator 2 (or any other mediators) (ca 250 mV vs Ag/AgCl). After a short time
(msec to sec), a steady state current will be measurable which is dependent only on the
concentration of mediator 1.

10 5. To measure Mediator 2 (Ferrocene)

A cathodic potential is applied to one set of fingers of the IDA which is capable of
reducing mediator 2 but not mediator 1 (ca 250 mV vs. Ag/AgCl). An anodic
potential is applied to the other set of fingers of the IDA which is capable of oxidizing
mediator 2 (ca 550 mV vs Ag/AgCl) . After a short time (msec to sec), a steady state
15 current will be measurable which is dependent only on the concentration of mediator 2.

Specific Binding Assay with Mixed Mediator Measurement.
Specific Assay of HbA1c in a blood sample

- 20 1. IDA electrodes are provided as in Paragraph A above.
2. Conjugates of mediators 1 and 2 and haptens or specific binding
members are provided using art-recognized procedures for covalent coupling using
either a homo-functional or hetero-functional linker. Specifically, a synthetic peptide
corresponding to the N-terminal sequence of the β -chain of HbA1c is conjugated to the
25 osmium complex. Similarly, a synthetic peptide corresponding to the N-terminal
sequence of HbA0 is conjugated to a second mediator, for example ferrocene.
3. Antibodies for the analytes (HbA1c and HbA0) which react
specifically with the N-terminal peptides which have been incorporated into the
conjugate are provided by standard methods for producing polyclonal antibodies. In
30 this case, sheep were immunized with carrier proteins to which were conjugated the
synthetic peptide sequences for HbA1c and HbA0. Following the appropriate
immunization schedule, the sheep were bled, and the antibody isolated from the blood
via ion exchange chromatography, followed by immunosorbent purification on a
column of the same N-terminal peptide with a different linker.

-25-

4. Appropriate stoichiometry of the reaction was determined for the two reactions independently by methods standard for immunoassay development. A solution containing a fixed amount of labeled conjugate was mixed with a solution with varying amounts of antibody, and, following an appropriate incubation period, the amount of free conjugate remaining was measured on the IDA electrode using the procedure described above. The amount of antibody just sufficient to achieve maximum inhibition of the conjugate (ca > 80%) was selected.

5. Reagent solution 1 was made containing a mixture of the two conjugates in the appropriate concentrations. Reagent solution 2 was made containing a mixture of the two antibodies in the amounts determined above. A blood sample was diluted ca 20-fold in a solution of 25mM citric acid/0.5% Brij-35. Following a 30 second incubation to allow for lysis and denaturation of the hemoglobin, to 66 uL of this diluted sample was added 33 uL of 1 M phosphate buffer, to adjust the pH back to neutral. 30 uL of antibody solution 2 was added, and the mixture allowed to incubate 30 sec. Then 30 uL of conjugate solution 1 was added, and the mixture measured on the IDA electrode. The concentration of HbA1c in the sample is related to the current measured from Mediator 1, and the concentration of HbA0 is related to the current from Mediator 2. The %HbA1c in the sample is related to the ratio of the measured amounts of Mediator 1 and Mediator 2.

20

Application to HbA1c Assay

Hemoglobin A1c is a specific glycohemoglobin in which the glycosylation takes place at the N-terminal of hemoglobin β -chain. The antibody binds specifically to HbA1c has an epitope sequence of Gluc-Val-His-Leu-Thr. To facilitate conjugation to other molecules, a nonnative amino acid has been added to the sequence, *e.g.*, Cys, Lys, or Lys-MH, to produce A1c peptides including: 1) Gluc-Val-His-Leu-Thr-Lys-MH; 2) Gluc-Val-His-Leu-Thr-Lys; 2) Gluc-Val-His-Leu-Thr-Cys.

HbA1c assay requires measuring both A1c concentration and total hemoglobin concentration and reports the results as a ratio of these two measurements (%HbA1c). It is advantageous to assay both A1c and total hemoglobin using same principle because ratioing would minimize biases due to environmental effects. Thus antibody has been raised to bind specifically to hemoglobins with unglycosylated N-

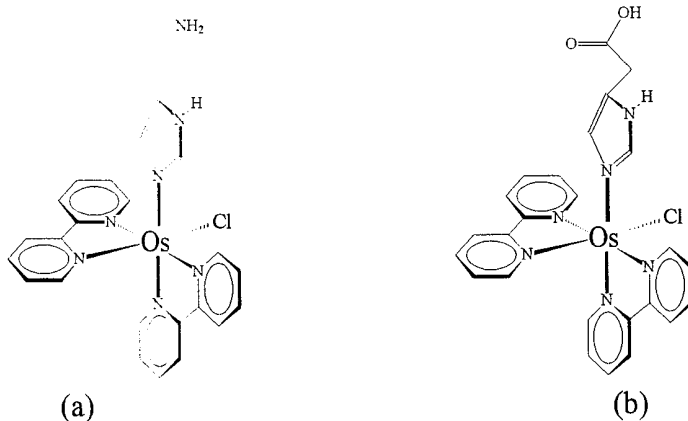
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terminus, *i.e.* with an epitope sequence of Val-His-Leu-Thr. Similarly, nonnative amino acid is added to the sequence to facilitate conjugation. The peptides used for total hemoglobin measurement is termed as A0 peptide. A0 peptides that have been used in the preparation of Os mediator-peptide conjugates include Val-His-Leu-Thr-
5 Cys and Val-His-Leu-Thr-Lys.

Conjugation Chemistry and Conjugates

There are many types of conjugation chemistry that can be employed to link Os mediator to a peptide. The following two conjugation chemistries employed
10 for the preparation of Os mediator-peptide conjugates have also been commonly used for preparing protein conjugates: 1) formation of amide bond by reactive ester with primary amine; 2) formation of thioether bond by maleimide with sulfhydryl group. Amide bond is preferred over thioether bond because amide bond is generally more stable. Based the preferred conjugation chemistry, the ligand on Os mediator can be
15 functionalized with either a primary amine group or a carboxylic acid group. The best location for these functional groups is believed to be the C-4 or C-5 positions on the imidazole ligand of Os mediator, however, functionalization through the non-Os-complexed imidazole ring nitrogen atom can also be carried out. Two different functionalized Os mediators were synthesized as described above.

20



25 Os mediator (a) was formed with histamine while Os mediator (b) was formed with imidazolacetic acid. However, it was found that the imino nitrogen of the

-27-

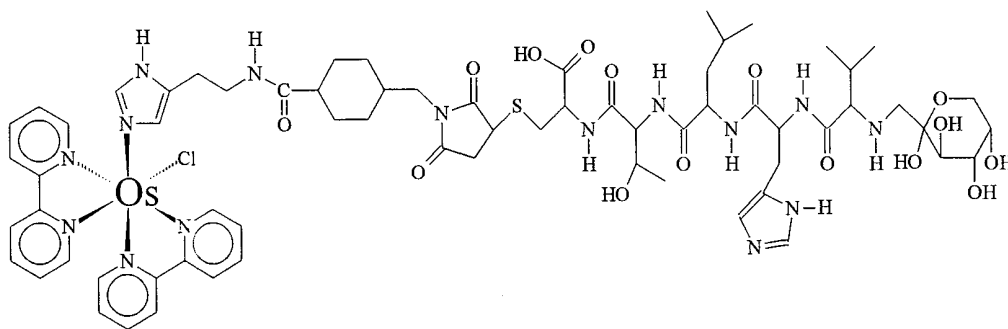
imidazole ring interferes with the activation of carboxylic acid group to reactive ester (*i.e.*, N-hydroxysuccinimide ester) using carbodiimide. Thus, use of carboxylic acid functionalized Os mediator in the synthesis of Os mediator-peptide conjugates gave much less favorable results.

5 The amine group on histamine ligand of Os mediator readily reacts with N-hydroxysuccinimide (NHS) ester to form amide bond. Two types of crosslinkers have been employed to link Os mediator to peptides, (a) heterofunctional crosslinker, having a NHS ester at one end and the other end has a maleimide or a sulfhydryl group; and (b) homofunctional crosslinker, *e.g.* both ends have NHS esters.

10 In the case of heterofunctional crosslinker, the crosslinker is first reacted with Os mediator with histamine ligand (Os histamine) at 1:1 molar ratio. One particular point needs to be noted here. Os mediator in oxidized form, *i.e.* Os(III), can instantly oxidize sulfhydryl group to form disulfide bond. It is important to keep Os center in the reduced form by addition of a small amount of reductant such as sodium
15 dithionite during the conjugation processes. The reaction progress can be monitored by analytical reverse-phase HPLC on a C18 column. Then the Os mediator-crosslinker adduct is isolated via preparative HPLC and the collected fraction is subsequently freeze-dried. Finally, the Os mediator-crosslinker adduct is reacted with the
20 appropriate peptide to form Os mediator-peptide conjugate. Again, the product is isolated by collecting appropriate fraction in preparative HPLC and the collected fraction is then freeze-dried.

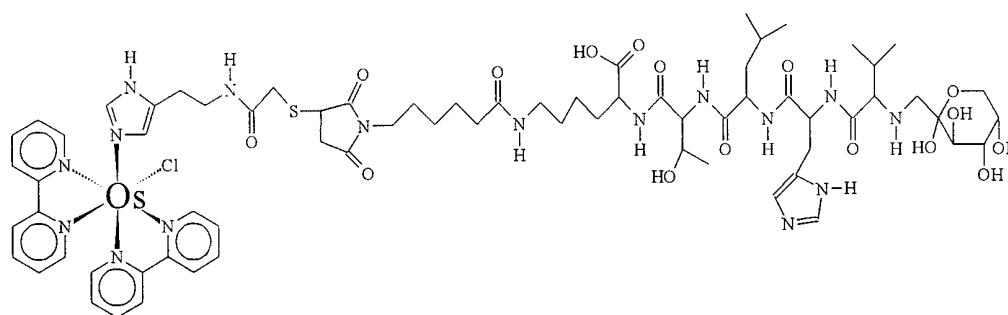
 Two different heterofunctional crosslinkers have been used for the synthesis of Os mediator-peptide conjugates. SMCC (succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate) is used for cystein-containing peptide,
25 while SATA (N-succinimidyl S-acetylthioacetate) is used for maleimide-containing peptide. Three Os mediator-peptide conjugates (two with A1c peptide and one with A0 peptide) have been made using heterofunctional crosslinkers and their structure are shown below: (a) Os-SMMC-A1c; (b) Os-SATA-A1c, and (c) Os-SATA-AO.

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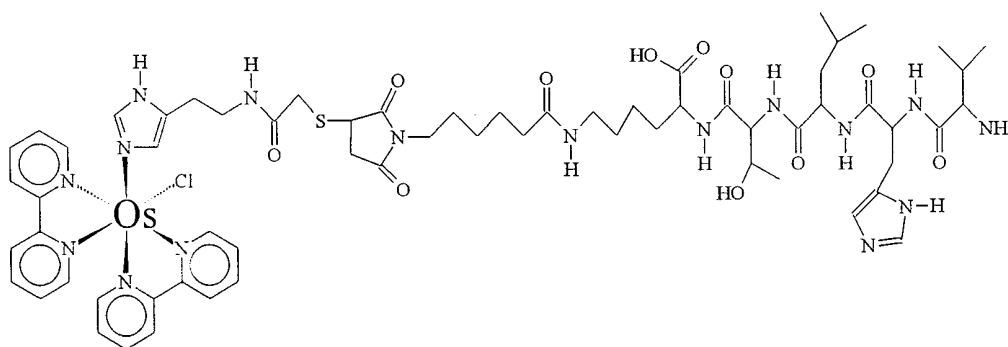
(a)

5



(b)

10



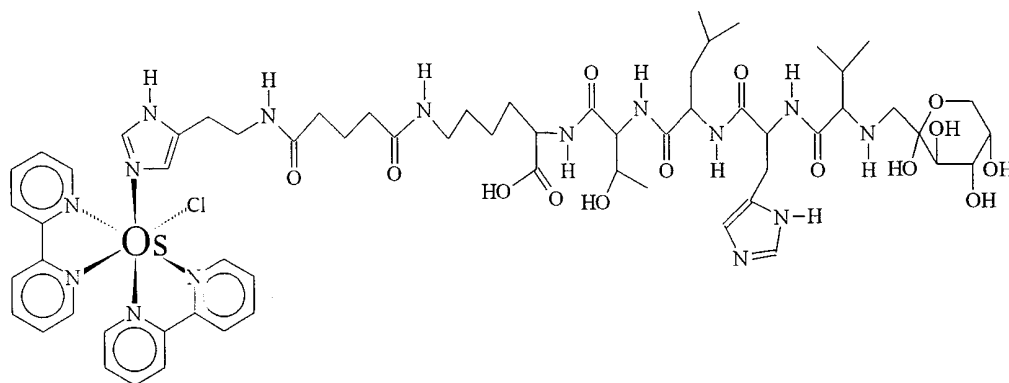
(c)

However, it has been found that these conjugates were not stable when they were stored as solutions. Analytical HPLC results indicated that these conjugates degrade. Mass spectroscopy confirmed that the instability is due to splitting of thioether bond present in these conjugates.

In order to avoid thioether bond in the conjugate, homofunctional crosslinker containing two NHS esters was used instead to prepare the conjugates.

The crosslinker used was DSG (disuccinimidyl glutarate). In order to prevent the formation of crosslinked Os mediator, *i.e.* Os-crosslinker-Os, a large excess of homofunctional crosslinker was used in the reaction with Os histamine at 4:1 molar ratio. Under this condition, only the desired product, *i.e.* Os-crosslinker, was formed.

- 5 The Os-DSG-A1c conjugate was similarly prepared using the procedure described earlier.



- 10 The preparation of analogous Os-DSG-A₀ conjugate requires an extra step since the unglycated N-terminal amine of HbA₀ peptide is also reactive toward NHS ester. In this case, the N-terminal amine of HbA₀ peptide is first protected by either a base-labile Fmoc¹ or an acid-labile Boc² group. After reacting with Os-DSG adduct to form Os-DSG-A₀ conjugate, the protecting group is then cleaved using
- 15 appropriate deprotection method (adding base for Fmoc or acid for Boc). The peptides prepared by solid-phase peptide synthesis already have N-terminal Fmoc protecting groups. The protecting groups are usually removed prior to cleavage of peptide from the resin beads, but they can also be left on if so desired. The HbA₀ peptide from Zymed has an intact Fmoc protecting group at N-terminus. Using this
- 20 strategy the Os-DSG-A₀ conjugate was successfully synthesized.

Many analytes cannot be assayed using enzyme-based sensors. They require the development of affinity biosensors or immunosensors which are based on the selective binding of antigens to antibodies. The key to the detection of this binding

¹Fmoc = 9-fluorenylmethyloxycarbonyl

²Boc = t-butyloxycarbonyl

event on electrochemical sensors is the inclusion of antigens labeled with redox labels. Bis(2,2'-bipyridyl) imidazolyl chloroosmium, a.k.a. Os mediator, possesses many properties that make it an excellent redox label for this purpose. In addition, a "handle" for linking it covalently to antigens can be added on without affecting its redox properties.

Several assay schemes can be used in affinity biosensors including, i) competitive binding assay (labeled antigen is competing for a limited number of binding sites); ii) sequential binding assays (labeled antigen is bound to excess binding sites); iii) heterogeneous assay (uses a separation step to separate bound and free labeled antigens); and iv) homogeneous assay (no separation step). The steps involved in a homogeneous sequential binding assay include binding the analyte to an antibody. The labeled antigen (analyte analog) binds to the remaining binding sites on the antibody. Finally the leftover free labeled antigen is detected at electrode surface. The resulting current will be a function of the amount of analyte present.

The detection of free labeled antigens can be achieved using either direct detection or amplified detection methods. Direct detection requires the use of advanced electrochemical techniques such as ac voltammetry, differential pulse voltammetry, square wave voltammetry or ac impedance in order to reach a sensitivity of 5 μ M or less. Amplified detection methods use dc amperometry with amplification through reaction with enzyme or chemical reductants or by using interdigital array (IDA) microelectrodes. The preferred detection method is amplified amperometry through cycling of free Os mediator label by using IDA microelectrodes in accordance with this invention.

25 **General Analytical HPLC Method For Osmium Conjugates**

All HPLC analysis were performed using a Beckman System Gold HPLC system consists of a 126 pump module and a 168 diode array detector module. Stationary phase is a Vydac analytical reverse-phase C18 analytical column. Other parameters are listed below.

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Mobil Phase: A = 0.1%TFA³ in H₂O
B = 0.1% TFA in CH₃CN

Flow rate: 1 mL/min

5

Gradient: 0- 5 min: 10% B
5-45 min: 10% B -> 50% B at 1%/min
45-50 min: 100% B

10 Detector: Channel A at 384 nm
Channel B at 220 nm.

Synthesis of Bis(2,2'-bipyridyl) dichloroosmium

15

1. Charge a 1 L one-neck RB flask with 19.335 grams K₂OsCl₆ (0.04019 mole) and 13.295 grams 2,2'-dipyridyl (0.08512 mole). Add 400 mL DMF to dissolve all reactants.

20

2. Heat the solution to reflux and then maintain reflux for 1 hour. Then turn off the heat and let solution cool to 30-40°C at ambient.

3. Filter the reaction mixture using a medium grade glass-frit filter. Rinse the flask with additional 20 mL DMF and wash the filter.

25

4. Transfer the filtrate into a 3-L beaker. Charge another 2-L beaker with 22.799 grams of NaS₂O₄ and dissolve in 2 L deionized water. Add this solution to the beaker containing Os/DMF filtrate dropwise using a dropping funnel. Keep the solution stirring at all time.

30

5. Then cool the mixture in an ice bath for at least 3 hours. Add ice as necessary.

35

6. Filter the mixture "cold" using a ceramic filter with filter paper. Wash the content on the filter with 50 mL, water twice and 50 mL ether twice.

40

7. Dry the product under high vacuum at 50°C overnight (at least 15 hours). Weigh the product and transfer into a brown bottle. Store in a desiccator at room temperature.

Typical yield = 16 gram or 70%.

Product is analyzed by UV-Visible spectroscopy and elemental analysis.

³TFA = trifluoroacetic acid.

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	<u>UV-Vis:</u>	Peak λ (nm)	ϵ ($M^{-1}cm^{-1}$)
		382	10,745
		465	9,914
		558	11,560
5		836	3,873

	<u>EA:</u>	C%	H%	N%	Cl%	Os%	H ₂ O%
10	Theoretical	41.89	2.81	9.77	12.36	33.17	0
	Actual	40.74	2.92	9.87	11.91		0.41

Synthesis of Bis(2,2'-bipyridyl) histamine chloroosmium

- 15 Charge a 2L one-neck RB flask with 11.3959 gram Os(bpy)₂Cl₂ (0.0198 mole) and 4.9836 gram histamine (0.0448 mole). Add 500 mL ethanol to dissolve the reactants. Then add 250 mL deionized water.
- 20 Heat the solution to reflux and maintain reflux for 6 hours. Let solution cool to RT at ambient.
- 25 Remove all ethanol using rotary evaporation. Then transfer the solution into a 500 mL beaker. Dissolve 2.136 gram NH₄BF₄ in 20 mL water. Add dropwise to Os solution. Precipitate forms. Cool in an ice bath for 30 min. Filter the mixture using a ceramic filter with filter paper. Wash the content on filter with ~20 mL water twice.
- 30 Dry under high vacuum at 50°C overnight (at least 15 hour).
- 35 Weigh the product and transfer to a brown bottle. Store in a desiccator at room temperature.

Typical yield = 7.6 gram or 52%.

35 Product is analyzed by UV-visible spectroscopy and HPLC.

	<u>UV-Vis:</u>	Peak λ (nm)	ϵ ($M^{-1}cm^{-1}$)
		355	7,538
		424	7,334
40		514	7,334
		722	2,775

HPLC: Elution time = 18.0 min
Purity by HPLC range from 65-85%

45

Preparation of [Os(bpy)₂(histamine)Cl]-heterofunctional crosslinker adduct

1. Weigh 0.1167 g [Os(bpy)₂(histamine)Cl]BF₄ (0.162 mmol) and transfer to a 5 mL Reacti-Vial. Add 1.0 mL DMF to dissolve the reactant. Add 25 μL triethylamine.
2. Add 0.0508g SMCC(0.150mmol)or 0.0390g SATA (0.168mmol). Stir the reactants at RT for 2 hours. Inject a sample into HPLC to monitor reaction progress.
3. If reaction is complete, dilute the solution with 0.1% TFA buffer to a final volume of 4.5 mL. Inject into preparative HPLC and collect the product peak.
4. Freeze dry the collected fraction overnight.
5. Weigh the product and transfer to a brown bottle. Store in a desiccated bag at -20°C.

Typical yield = 40 mg or 25%.

Product is analyzed by HPLC and ES/MS.

	<u>HPLC</u>	<u>ES/MS</u>
Os-SMCC:	Elution time = 32.1 min	m ⁺ /e = 434.2
Os-SATA:	Elution time = 27.5 min	m ⁺ /e = 382.8

Preparation of [Os(bpy)₂(histamine)Cl]-homofunctional crosslinker adduct

1. Weigh 0.2042 g DSG (0.626 mmol)) and transfer to a 5 mL Reacti-Vial. Add 0.75 mL DMF to dissolve the reactant.
2. Weigh 0.1023 g [Os(bpy)₂(histamine)Cl]BF₄ (0.142 mmol) and transfer to a separate 5 mL Reacti-Vial. Add 1.0 mL DMF to dissolve the reactant. Add 25 μL triethylamine. Then add Os/DMF solution dropwise to DSG/DW solution with constant stirring. After reacting for 2 hours at RT, inject a sample into HPLC to monitor reaction progress.
3. If reaction is complete, dilute the solution with 0.1% TFA buffer to a final volume of 4.5 mL. Inject into preparative HPLC and collect the product peak.
4. Freeze dry the collected fraction overnight.

5. Weigh the product and transfer to a brown bottle. Store in a desiccated bag at -20°C.
 Typical yield = 45 mg or 35%.

5 Product is analyzed by HPLC and ES/MS.

	<u>HPLC</u>	<u>ES/MS</u>
Os-DSG:	Elution time = 27.1 min	m ⁺ /e = 429.2 and 859.6

10

Preparation of Os-SATA-Alc Conjugate

- 15 1. Weigh 40.5 mg Os-SATA (0.0529 mmol) and transfer to a 5 mL Reacti-Vial with stir bar. Add 1.0 mL PBS (pH 7.5) to dissolve. Add 20 mg Na₂S₂O₄ in order to keep Os in reduced form.
- 20 2. Add 1.0 mL deacetylation buffer (PBS pH7.5 + 0.5 M hydroxylamine and 25 mM EDTA) to deprotect the sulfhydryl group. Inject a sample into analytical HPLC to determine whether deprotection is complete by appearance of a new peak at 25.8 min.
- 25 3. Add 45 mg HbAlc-MH peptide (0.0474 mmol) and let react at RT for 1 hour. Inject a sample into analytical HPLC to monitor reaction progress.
- 30 4. If reaction is complete, dilute the mixture with 0.1 %TFA buffer to a final volume of 4.5 mL. Inject into preparative HPLC to collect product peak.
5. Freeze dry the collected fraction overnight (at least 15 hour).
- 35 6. Weigh the product and transfer to a brown bottle. Store in a desiccated bag at -20°C.

35

Typical yield = 12 mg

Product is analyzed by HPLC and ES/MS.

40

	<u>HPLC</u>	<u>ES/MS</u>
Os-SATA-Alc:	elution time = 27.6 min	m ⁺ /e = 559.1 and 838.5

45

Preparation of Os-SMCC-Alc Conjugate

1. Weigh 39.0 mg Os-SMCC (0.0452 mmol) and transfer to a 5 mL Reacti-Vial with stir bar. Add 1.0 mL PBS (pH 6.0) to dissolve.
2. Add 30.0 mg Hblc-Cys peptide (0.0450 mmol). Let reaction proceed at RT for 2 hours. Inject a sample into analytical HPLC to monitor reaction progress.
3. If reaction is complete, dilute the mixture with 0.1% TFA buffer to a final volume of 4.5 mL. Inject into preparative HPLC to collect product peak.
4. Freeze dry the collected fraction overnight (at least 15 hour).
5. Weigh the product and transfer to a brown bottle. Store in a desiccated bag at -20°C.

Typical yield = 12 mg

Product is analyzed by HPLC and ES/MS.

	<u>HPLC</u>	<u>ES/MS</u>
Os-SMCC-Alc:	elution time = 27.6 min	$m^+/e = 480.5$ and 534.4

Preparation of Os-SMCC-Ao Conjugate

1. Weigh 37.0 mg Os-SMCC (0.0426 mmol) and transfer to a 5 mL Reacti-Vial with stir bar. Add 1.0 mL PBS (pH=6.0) to dissolve.
2. Add 24.3 mg HbAo-Cys peptide (0.0425 mmol). Let reaction proceed at RT for 2 hours. Inject a sample into analytical HPLC to monitor reaction progress.
3. If reaction is complete, dilute the mixture with 0.1%TFA buffer to a final volume of 4.5 mL. Inject into preparative HPLC and collect the product peak.
4. Freeze dry the collected fraction overnight (at least 15 hour).
5. Weigh the product and transfer to a brown bottle. Store in a desiccated bag at -20°C.

Typical yield = 15 mg

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Product is analyzed by HPLC and ES/MS.

	<u>HPLC</u>	<u>ES/MS</u>
5 Os-SMCC-A ₀ :	elution time = 27.9 min	m ⁺ /e = 360.7 and 720.5

Preparation of Os-DSG-Alc Conjugate

10

1. Weigh 32.0 mg Os-DSG (0.037 mmol) and transfer to a 5 mL Reacti-Vial with stir bar. Add 0.75 mL DMF to dissolve. Add 25 μ L triethylamine.
- 15 2. Add 26.5 mg HbAlc-Lys peptide (0.0349 mmol). Let reaction proceed at RT for 2 hours. Inject a sample into analytical BPLC to monitor reaction progress.
- 20 3. If reaction is complete, dilute the mixture with 0.1%TFA buffer to a final volume of 4.5 mL. Inject into preparative HPLC and collect the product peak.
4. Freeze dry the collected fraction overnight (at least 15 hour).
- 25 5. Weigh the product and transfer to a brown bottle. Store in a desiccated bag at -20°C.

Typical yield = 16 mg

30 Product is analyzed by HPLC and ES/MS.

	<u>HPLC</u>	<u>ES/MS</u>
35 Os-DSG-Alc:	elution time = 23.5 min	m ⁺ /e = 501.8 and 752.8

Preparation of Os-DSG-A₀ Conjugate

- 40 1. Weigh 52.0 mg Os-DSG (0.0605 mmol) and transfer to a 5 mL Reacti-Vial with stir bar. Add 1.0 mL DMF to dissolve. Add 25 μ L triethylamine.
- 45 2. Add 49.1 mg Fmoc-HbA₀ peptide (0.0606 mmol). Let reaction proceed at RT for 2 hours. Inject a sample into analytical HPLC to monitor reaction progress by the appearance of peak at 40.3 min for Os-DSG-A₀(Fmoc).

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3. If reaction is complete, inject additional 100 μ L triethylamine. After 1 hour, inject sample into analytical HPLC to determine whether all Fmoc protection group is removed by disappearance of the peak at 40.3 min.
- 5 4. If removal of Fmoc is complete, dilute the mixture with 0.1%TFA buffer to a final volume of 4.5 mL. Inject into preparative HPLC to collect product peak.
- 10 5. Freeze dry the collected fraction overnight (at least 15 hour).
6. Weigh the product and transfer to a brown bottle. Store in a desiccated bag at -20°C .

Typical yield = 16 mg

15

Product is analyzed by HPLC and ES/MS.

	<u>HPLC</u>	<u>ES/MS</u>
20 Os-DSG-A ₀ :	elution time = 23.2 min	m ⁺ /e = 447.4 and 670.3

Synthesis of bis (4,4'-dimethyl-2,2'-bipyridyl) 4-methyl-4'-carboxylpropyl-2,2'-bipyridyl osmium [Os(dm-bpy)₂(mcp-bpy)]Cl₂

25 Potassium hexachloroosmium was reacted with 4,4'-dimethyl-2,2'-dipyridyl at 1:2 molar ratio by refluxing in DMF. The potassium chloride precipitate was filtered and the dimethyl-bipyridyl dichloroosmium complex was reduced from +3 oxidation state to +2 oxidation state using excess sodium dithionite. The product was recrystallized in DMF/water mixture at 0° and recovered by filtration.

30 4,4'-Dimethyl-2,2'-bipyridyl dichloroosmium was reacted with 4-methyl-4'-carboxylpropyl-2,2'-dipyridyl by refluxing in ethylene glycol. The solvent was removed by rotary evaporation. The product was dissolved in DMF and precipitate in ethyl ether. The product was dried in a vacuum oven overnight.

35 Analyticals: Product and intermediate product were analyzed by HPLC and mass spectroscopy for purity and identity of the compound.

Os(dm-bpy)₂Cl₂: Theoretical MW = 629.6, MS showed 8 isotope peaks with most abundant peak at 630. HPLC elution time at 29.94 min with a purity of 90%+

40 Os(dm-bpy)₂(mcp-bpy): MS confirmed the MW at 814.5 and HPLC showed a purity greater than 85%.

Synthesis of biotin-Os complex conjugate Biotin-Os(dm-bpy)₂(mcp-bpy)]Cl₂

The carboxyl group was activated by reacting the above Os complex with dicyclohexylcarbodiimide in the presence of N-hydroxysuccinimide. The active ester Os complex was isolated using preparative HPLC method and then reacted with amine-containing biotin to form the final conjugate.

Experiment to independently measure the concentration of two electroactive conjugate species.

Os(bipy)HisCl-DSG-HbA1c was prepared as described above.

Ferrocene-AMCHA-DADDO-biotin was prepared from ferrocene monocarboxylic acid, the crosslinker aminomethylcyclohexylic acid, the chain extender 1,8-diamino-3,6-DiOxoOctane and biotin as described elsewhere.

Mixtures of the two conjugates were prepared to evaluate the ability of the method of the invention to independently measure the concentration of the conjugates, and make corrections for variations in reagent amounts, electrode response, and environmental conditions.

Part 1: Simple Mixed conjugate response

The following matrix of solutions was prepared in 10 mM phosphate buffer with 150 mM NaCl and 0.5 % Brij-35, a non-ionic surfactant.

Os-DSG-HbA1c uM/l	Ferrocene-Biotin uM/l	Os-DSG-HbA1c uM/l	Ferrocene-Biotin uM/l
0	0	0	12.5
6.25	0	6.25	12.5
12.5	0	12.5	12.5
25	0	25	12.5

Os-DSG-HbA1c uM/l	Ferrocene-Biotin uM/l	Os-DSG-HbA1c uM/l	Ferrocene-Biotin uM/l

0	25	0	50
6.25	25	6.25	50
12.5	25	12.5	50
25	25	25	50

5

An Interdigitated array (IDA) microelectrode was fabricated according to the procedures described. In addition to the IDA, the chip had a silver/silver chloride electrode on the surface to function as the reference electrode and counter electrode. This electrode was produced with the same lithographic process, and then electroplated with silver and silver chloride according to standard techniques. The IDA was connected to a bipotentiostat capable of controlling the potential relative to the reference and measuring the current at each of the electrodes of the IDA. Aliquots of the solutions were placed onto the surface of the chip, such that the IDA and the reference electrodes were covered.

Measurements were made by first applying -100 mV (vs. Ag/AgCl) to one electrode of the IDA, and 200 mV to the other electrode for a period of 30 seconds. At this time, current was measured at each electrode. Then 200 mV was applied to one electrode, and 550 mV to the other. After 30 seconds, current was again measured. See Figure 9 for a summary of the results, which clearly demonstrate that the concentrations of the two mediators can be independently measured with this method.

Part 2. Concentration Co-variance of Dual Mediators on IDA electrodes.

In this experiment, it was demonstrated that by making a mixture of known concentrations of two different mediators, and measuring different dilutions of that mixture by the method of the invention, the ratio of the concentrations of the mediators remains constant. (Internal standard application).

The same two mediator conjugates were used as in Part 1. (Os-DSG- A1c and Fc-Bi)

From a solution containing 40 μ M of each conjugate, solutions containing 27 μ M, 32 μ M, and 36 μ M of each conjugate were prepared in the same buffer (PBS/Brij).

30

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The solutions were measured as in the previous example. Each solution was measured on 5 different IDA electrodes. The results are presented as the means, standard deviations, and coefficient of variation for each solution separately, and for all solutions pooled over all electrodes.

5

Individual concentrations		Os-DSG-A1c	Fc-Bi	Os/FC
28 uM	Mean	156	85	1.84
	S.D.	17.5	13.2	0.08
	% C.V.	11.2	15.5	4.4
32 uM	Mean	165	88	1.88
	S.D.	11	7.2	0.04
	% C.V.	6.7	8.2	2.0
36 uM	Mean	189	102	1.85
	S.D.	12.5	6.9	0.04
	% C.V.	6.6	6.7	2.3
40 uM	Mean	208	110	1.90
	S.D.	9.5	5.5	0.06
	% C.V.	4.6	5.0	3.4
Pooled Concentrations	Mean	182	97	1.87
	S.D.	24.4	13.2	0.06
	% C.V.	13.4	13.5	3.4

This example clearly demonstrates that the internal standard effect of measuring two conjugates or mediators and calculating the ratio gives significantly improved precision of measurement, not only within each solution (compensation for variation between electrodes) but over all solutions (compensation for variation in sample dilution or amount).

30 Part 3. Temperature compensation

It was desired to show the effectiveness of the method in compensating for environmental influences such as Temperature variation on the accuracy or the measurement.

The same two conjugates were prepared in solution at 40 uM as before. They were measured as before on IDA electrodes, either at room temperature or warmed to 35-40 C on a heated metal plate prior to the measurement. The solutions were also warmed to 37 C prior to application to the electrodes.

	Room Temperature (23C)	Warmed (35-40) C	Ratio of response Warm / RT
Os-DSG-A1c	261	387	1.45
Fc-Bi	179	268	1.5
Ratio Os/Fc	1.46	1.44	0.99

5

As demonstrated by the results, the measured values increase by almost 50 % in the case of the warmed samples, which would lead to a large measurement error. However the use of the internal standard and ratio calculation effectively eliminates the temperature dependence of the result.

10

Immunoassay Detection of HbA1c with Osmium Mediator Conjugates.

The goal of all diabetic therapy is to maintain a near normal level of glucose in the blood. Home blood glucose kits are available to monitor the current glucose values and are valuable for diabetics to adjust day to day insulin doses and eating habits. Unfortunately, since the tests only measures a point in time result, it does not tell them the overall effectiveness of their actions in maintaining glycemic control. Measurement of glycosylated hemoglobin is now becoming widely accepted as an index of mean blood glucose concentrations over the preceding 6-8 weeks and thus provides a measure of the effectiveness of a diabetic's total therapy during periods of stable control. Since monitoring a diabetic's glycated hemoglobin can lead to improved glycemic control, the ADA recommends routine measurements of four times a year up to once a month for less stable type I diabetics.

Several technologies are available for the measurement of glycated hemoglobin. They include immunoassays for HbA1c (TinaQuant, BMC; DCA2000, Ames; and Unimate, Roche), ion exchange (Variant, BioRad; Eagle Diagnostics), and affinity chromatography (ColumnMate, Helena; GlyHb, Pierce).

One objective of this project is to develop a simple to use disposable strip for electrochemical detection of HbA1c for use in both physician offices and the home.

30

The most significant parameter for assessing patient condition is ratio of HbA1c to HbA₀, and thus the measurement of both glycated (HbA1c) and nonglycated (HbA₀) values is required to calculate the ratio. This requires two separate measurements. It is preferable to use the same technology to measure both the

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glycated and nonglycated fractions, thus removing some sample and environmental interferences. Measurement of HbAlc via electrochemical immunoassay is described below. Electrochemical HbA₀ immunoassay measurements are carried out using the same methods as that for HbAlc. The concentrations of HbA₀ are significantly higher.

5 One alternative to A₀ measurements using immunoaffinity would be to measure total hemoglobin directly using biamperometry or differential pulse voltammetry. This can be easily accomplished since hemoglobin is readily oxidized by $[Os(bpy)_2(im)Cl]^{2+}Cl_2$.

The N-terminal valine of the β -chain of hemoglobin A is the site of glycosylation in HbAlc, and serves as a recognition site for the antibody. In whole
10 blood the N-terminal valine is not accessible for the antibody to bind. Access is gained by lysing the red cells to release the hemoglobin followed by a conformation change (denaturing or unraveling) to adequately expose the HbAlc epitope. Dilution of the sample may occur as part of the lysing/denaturing process or may be required post denaturing to prepare the sample for the antibody (adjust pH, other) or bring the
15 sample into a range suitable for electrochemical immunoassay. In one embodiment, a fixed amount of antibody is incubated with the prepared sample and it binds to the HbAlc epitopes of the sample. The free antibody and the antibody bound sample is then combined with the osmium peptide conjugate (Alc or A₀) to allow the remaining unbound antibody to bind to the mediator label. When the mediator is bound to the
20 antibody (a macromolecule), it can not freely diffuse to interact with the electrode and thus currents generated are significantly reduced. The remaining unbound mediator label is therefore proportional to the concentration of HbAlc in the sample. The unbound mediator can be measured electrochemically preferably using an interdigitated array electrode with bipotentiostatic control.

25 Blood lysis is necessary to release the hemoglobin followed by denaturing to expose the HbAlc epitope. Lysis can easily be accomplished via surfactants, osmotic effects of dilution with water, and directly by many denaturants. Blood lysed through a freeze/thaw cycle was shown not to significantly interfere with the biamperometric measurement ("open rate" with and without lysed blood was
30 almost identical). Conversely, denaturing the lysed blood with a variety of known denaturants to expose the HbAlc epitope has shown significant suppression of the electrochemical response, inhibiting measurement of an HbAlc dose response. Only

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LiSCN and citric acid from the list of evaluated denaturants shown in Table 1 was able to expose the Alc epitope and minimize protein fouling enough to measure an HbAlc dose response.

Denaturing the sample for antibody recognition without severely
5 fouling the electrode surface is important for successful development of an HbAlc immunoassay. Although LiSCN has been used almost exclusively to show feasibility, it has many limitations that would hinder its use in the disposable. Citric acid, a solid at RT may offer benefits as a denaturant if it could be dried onto a strip followed by a diluent to adjust the pH to neutral. Acid or base blood denaturing followed by a final
10 pH adjustment with a buffered diluent is an area worth further evaluation. One problem that was initially encountered was precipitation in adjusting the pH back to neutral, which can be overcome by using a different buffer or with the addition of surfactants.

15

Table 1. Blood Denaturants

Method	Comments
KSCN	<ul style="list-style-type: none"> Initial work did not show a dose response with KSCN denatured blood. KSCN has a larger negative effect on the electrochemical response than LiSCN. Literature shows that LiSCN is more effective than KSCN (concentrated efforts on LiSCN).
5 DCA2000 Buffer	<ul style="list-style-type: none"> Denaturing of blood not evident with the higher blood concentrations required for this assay. Higher concentrations of LiSCN are shown below.
LiSCN	<ul style="list-style-type: none"> Method used by Ames DCA2000 HbAlc immunoassay.
10 Citric, Sulfuric, Hydrochloric, & Perchloric Acid	<ul style="list-style-type: none"> Blood HbAlc dose response (high/low) was seen with citric acid and was comparable to the response with LiSCN Evidence of blood denaturing was seen by all: "solution turned brown." Citric acid is preferred. Adjustment of pH to neutral after denaturing also saw problems of precipitation. Enzyme mediated responses with Gluc-Dor at pH 5.7 reduces response 50% compared to pH 7-8. Citric acid blood denaturing method is shown in Figure 5.
Pepsin/Citric Acid	<ul style="list-style-type: none"> Roche HbAlc immunoassay uses pepsin/citric acid to hemolyze and proteolytically degrade hemoglobin to glycoproteins accessible by the antibody. Denaturation was apparent by the color change to a brownish red solution. Hemoglobin Alc dose response (high/low) was obtained comparable to LiSCN and citric acid denaturants. The procedure was identical to that of citric acid used above with the exception of pepsin added to the acid. Results were identical to that of citric acid.
15 TTAB (Tetra decyltrimethyl ammonium bromide)	<ul style="list-style-type: none"> Method of denaturing used in the TinaQuant HbAlc turbidimetric immunoassay. Evidence of denaturing: "solution turned green" TTAB concentrations 0.0125-0.2% severely suppressed enzyme mediated (Glucdor/PQQ/Glucose) biamprometric measurements. Open rates were 16-50 nA compared to 140 nA without TTAB.
NaOH	<ul style="list-style-type: none"> Evidence of denaturing: "solution turned brown." NaOH does not adversely effect the enzyme mediated electrochemistry. Even at high pH the open rates do not change, although pH adjustment will probably be required to bring it within an optimal range for the antibody. NaOH denatured blood suppresses the open rates probably due to protein fouling. Lowering the pH to neutral tends to cause some precipitation.

Table 2. Effective Blood Denaturing Procedure for 2% Blood

	LiSCN (One Step)	LiSCN (Two Step)	Citric Acid (2 Step)
5	40 μ L 6 M LiSCN 20 μ L 5% Tween in PBS 20 μ L Blood Mix (vortex) and allow to denature for 10 minutes. Dilute with 920 μ L DI H ₂ O.	960 μ L 1.5 M LiSCN 20 μ L 5% Tween in PBS 20 μ L Blood Mix (vortex) and allow to denature for 10 minutes.	200 μ L 0.2 M Citric Acid 20 μ L 5% Tween in PBS 20 μ L Blood Mix (vortex) and allow to denature for 10 minutes. Dilute with 760 μ L 8X PBS (0.1% Tween)
10	Denaturing time was not optimized. Limited data supports longer times for better precision using this method.	Denaturing time was not optimized. Data indicates shorter times may be adequate.	No optimization studies were performed.

PBS = 10 mM Phosphate Buffer, 2.7 mM KCl, 137 mM NaCl pH=7.4

15 Increased level of surfactant (5% Tween) reduces or eliminates precipitate.

Electrode fouling caused by denatured blood proteins adsorbing to the electrode surface can impede electron transfer and thus decrease electrode sensitivity.

20 Electrode fouling or passivation occurs more or less immediately following sample contact with the surface thus minimizing the severity of denaturing in the sample should be the first approach. Surface conditions that are hydrophobic will favor adhesion of the proteins and thus fouling may be minimized with electrode surfaces of higher surface energies. This explains why gold electrodes shows less fouling with

25 denatured blood than palladium. Reduction of protein fouling may be achieved by changing or protecting the electrode surface. Modifications that make the surface more hydrophilic should reduce the amount of fouling and can be accomplished by argon or oxygen plasma treatment or corona treatment. Selective coatings that could block the proteins from reaching the electrode surface can usually partially circumvent

30 the problem have been used in the field to reduce fouling. Unfortunately, dramatic decreases in responses greater than seen with the denatured blood proteins are normally noted with their use. Hydrophilic coatings such as PEO were also evaluated and showed some improvement, but have similar problems of decreased magnitude and precision caused by forcing reagents to diffuse through the polymers. Reagents

35 dispensed and dried over the electrodes may help reduce the magnitude of protein fouling with less negative effects.

Mediator concentration dose response, inhibition with antibody and reversal with a BSA-Alc polyhaptan were evaluated and summarized in Table 3. The

Os-DSG-Alc is stable in a lyophilized form and when frozen in solution at -20°C (40 and 80 μM).

Table 3. Osmium Mediator Labels

Mediator Label	Concentration Response	Inhibition with Antibody	Reversal	Comments
Os-SMCC-Alc	Linear	PAB IS ($\leq 92\%$) PAB DE ($\leq 97\%$) MAB ($\leq 50\%$)	Yes with BSA-Alc polyhaptan	% =Inhibition values ranged from 16% to 97% depending on age of Os-SMCC stock solution. Degrades in solution.
Os-SATA-Alc	Linear	PAB IS ($\leq 44\%$)	Yes with BSA-Alc polyhaptan	Stability similar to SMCC.
Os-DSG-Alc	Linear	PAB IS ($\leq 91\%$) PAB DE ($\leq 87\%$) MAB ($\leq 78\%$)	Yes with BSA-Alc polyhaptan	More stable than conjugate made with SATA and SMCC crosslinker but still degrades in solution.
Os-SATA-A ₀	Linear	Yes with Sheep B<HbA ₀ > ($\leq 84\%$). No with Zymed rabbit antibodies	No with A ₀ HB-peptide#1	A ₀ conjugate was found to be unstable in solution. Conjugate was not lyophilized.

Polyclonal DE (ion-exchange) purified sheep antibody is used in the TinaQuant HbA1c assay. IS (immunosorbent) antibody is prepared using standard IS purification methodology. Samples of a monoclonal antibody were also obtained for evaluation. Inhibition curves were performed in solution with all mixing occurring in microcentrifuge tubes. Assays were measured by applying 20 μL onto 6 mm² palladium electrodes with the conditions shown in Table 3. Inhibition curves with the three hemoglobin A1c antibodies (PAB IS, PAB DE, and MAB) were generated by fixing OS-DSG-A1c at 5 μM and varying the antibody concentration. Both PAB IS and MAB showed the expected stoichiometric relationship for inhibition with the osmium peptide conjugate indicating efficient and fast binding of the antibody to the A1c peptide. The polyclonal IS and monoclonal both showed steep inhibition curves with maximum change being reached close to 5 μM . Additional antibody above 5 μM showed little effect on increasing the inhibition. The less purified PAB DE antibody had a much smaller slope and as expected required more than 3 times the amount to get close to maximum inhibition. Figure 6 shows the inhibition curves for each of the

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HbA1c antibodies tested. From the inhibition curves we were able to select reasonable concentrations of antibody for maximum reversal with Alc samples.

Inhibition curves were also performed for the Os-SMCC-Alc (Max = 97%) and OS-SATA-Alc (Max = 44%) mediator labels. Stability of the mediator labels were also evaluated by monitoring % inhibition values over time. All of the
5 mediator labels showed some degradation when stored in dilute solutions (40 μ M) at RT. Samples frozen at -20°C appear to be stable.

For demonstrating inhibition reversal, antibody concentrations of 4 μ M for both PAB IS and MAB and 15 μ M for PAB DE were chosen from the inhibition
10 curves shown above. Reversal curves were then generated using a series of dilutions of BAS-A1c polyhaptan with a ~1:1 Alc:BSA. The BSA-A1c acts as our sample and binds to the antibody. Figure 10 shows the reversal curves for the three antibodies.

While these feasibility studies for a HbA1c immunoassay used an enzyme mediated amplification method (Glucdor/PQQ/glucose was used to regenerate
15 reduced mediator after oxidation at the electrode surface providing a higher diffusion controlled current is given by the cottrell equation), they are considered to be indicative of results attainable with the use of IDA electrodes with bipotentiostatic control is most preferred for measuring mediator labeled conjugates in accordance with this invention.

20

1. A method for measuring the concentration of one or more analytes in a liquid sample, said method comprising
- contacting a volume of said liquid sample with
- 1) predetermined amounts of at least a first and second redox reversible species, each respective species having a redox potential differing by at least 50 millivolts from that of each other species, at least one species comprising a liquid sample diffusible conjugate of a ligand analog of an analyte in the liquid sample and a redox reversible label, said conjugate capable of competitive binding with a specific binding partner for said analyte, and
 - 2) a predetermined amount of at least one specific binding partner for each analyte to be measured; and
- electrochemically determining the concentration of each of said diffusible redox-reversible species in the liquid sample by
- contacting said sample with an electrode structure including a reference electrode and at least first and second working electrodes dimensioned to allow diffusional recycling of the diffusible redox reversible species in the sample when a predetermined redox-reversible-species-dependent cathodic potential is applied to one working electrode and a predetermined redox-reversible-species-dependent anodic potential is applied to a second working electrode, said diffusional recycling of said species being sufficient to sustain a measurable current through said sample,
- applying a first cathodic potential to the first working electrode and a first anodic potential to the second working electrode, said first cathodic and anodic potentials corresponding to those respective potentials necessary to establish current flow through the sample due to diffusional recycling of the first redox reversible species without significant interference from said second redox reversible species,
- measuring current flow at said first anodic and cathodic potentials,
- applying a second cathodic potential to said first or second working electrode and a second anodic potential to the other working electrode, said second cathodic and anodic potential corresponding to those respective potentials necessary to establish current flow through the sample due to diffusional recycling of

the second redox-reversible-species without significant interference from the first redox reversible species,

measuring current flow at said second anodic and cathodic potentials, and

5 correlating the respective measured current flows to that for known concentrations of the respective diffusible redox reversible species.

2. The method of claim 1 wherein the cathodic and anodic potentials are applied to the working electrodes using a bipotentiostat.

3. The method of claim 1 wherein the redox reversible label is a metal ion
10 complex selected from ferrocene and nitrogen-coordinated complexes of transition metal ions.

4. The method of claim 1 wherein the redox reversible label is a redox reversible organic group.

5. The method of claim 1 for measuring the concentration of two analytes
15 in a liquid sample wherein the respective redox potentials of the first and second redox-reversible-species differ by at least 100 millivolts.

6. The method of claim 1 for measuring the concentration of one or more analytes in a liquid sample wherein current flow is measured as at least one of the anodic or cathodic potentials is held at the predetermined value and the potential of the
20 other is swept through its predetermined value.

7. The method of claim 1 for measuring two proteinaceous analytes in a liquid sample wherein the ligand analog component of the first redox-reversible-species is a peptide comprising an epitope of a first analyte and the ligand analog component of a second redox-reversible-species is a peptide comprising an epitope of a second
25 analyte.

8. The method of claim 7 wherein one specific binding partner is an antibody recognizing the epitope of the first analyte and the other specific binding partner is an antibody recognizing the epitope of the second analyte.

9. The method of claim 1 for measuring one analyte in a liquid sample
30 wherein the respective ligand analog component of the first and second redox-reversible-species are different ligand analogs of a single analyte.

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10. The method of claim 9 wherein the ligand analog component of the first redox reversible species is a peptide comprising a first epitope of the analyte, and the ligand analog component of the second redox-reversible-species is a peptide comprising a second epitope of the analyte, and the specific binding partners are first and second antibodies each recognizing the respective first and second epitopes.

11. A device for detecting or quantifying one or more analytes in a liquid sample, said device comprising

at least two redox reversible species, each capable of diffusion in said liquid sample at least in the presence of a respective predetermined analyte, said redox reversible species having respective redox potentials differing by at least 50 millivolts,

an electrode structure for contact with the liquid sample, said electrode structure including a reference electrode and working electrodes dimensioned to allow diffusional recycling of a diffusible redox reversible species in the liquid sample in contact with the electrode structure when a predetermined redox-reversible-species-dependent cathodic potential is applied to one working electrode and a predetermined redox-reversible-species-dependent anodic potential is applied to a second working electrode, said diffusional recycling of said species being sufficient to sustain a measurable current through each working electrode, and

conductors communicating with the respective electrodes for applying said anodic potential and said cathodic potential and for carrying the current conducted by the electrode.

12. The device of claim 11 further comprising a sample chamber, said wherein chamber having a sample receiving port and dimensioned so that it fills by capillary flow when the liquid sample is contacted with the sample receiving port.

13. The device of claim 11 wherein the electrode structure comprises microarray electrodes selected from the group consisting of arrays of microdiscs, microbands or microholes.

14. The device of claim 11 wherein the electrode structure comprises interdigitated microarray electrodes.

15. The device of claim 11 wherein the redox reversible species are located for contact with the liquid sample as it is delivered to the chamber.

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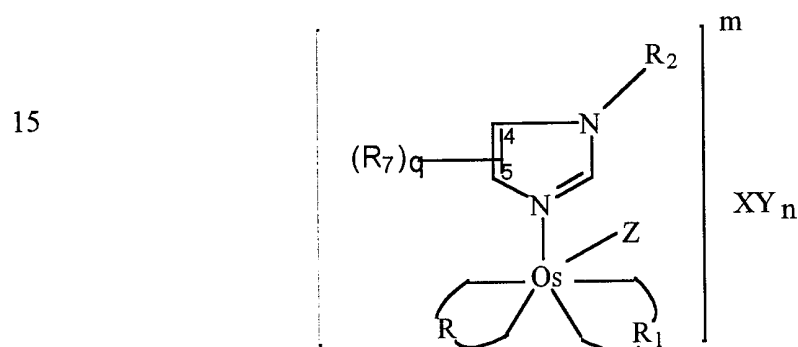
16. The device of any of claims 11 wherein at least one redox reversible species includes an osmium complex.

17. The device of claim 11 wherein at least one of the redox reversible species comprises ferrocene or a redox reversible derivative thereof.

5 18. The device of claim 11 wherein two redox reversible species are positioned for contact with the liquid sample as it is delivered to the chamber and each species is an osmium complex.

19. The device of claim 11 including at least one redox reversible species comprising ferrocene or a redox reversible derivative thereof and at least one redox
10 reversible species comprising an osmium complex.

20. The device of claim 11 wherein at least one of the redox-reversible species is An electrochemically detectable compound of the formula



wherein

R and R₁ are the same or different and are 2,2'-bipyridyl, 4,4'-disubstituted-2,2'-bipyridyl, 5-5'-disubstituted-2,2'-bipyridyl, 1,10-phenanthrolyl, 4,7-disubstituted-1,10-phenanthrolyl, or 5,6-disubstituted-1,10-phenanthrolyl,

25 wherein each substituent is a methyl, ethyl, or phenyl group,

R and R₁ are coordinated to Os through their nitrogen atoms;

q is 1 or 0;

R₇ is B-(L)_k-Q(CH₂)_i-;

R₂ is hydrogen, methyl, or ethyl when q is 1, and R₂ is B-(L)_k-Q(CH₂)_i-

30 when q is 0;

wherein in the group B-(L)_k-Q(CH₂)_i-

Q is O, S, or NR₄ wherein R₄ is hydrogen, methyl or ethyl;

-L- is a divalent linker;

k is 1 or 0;

i is 1, 2, 3, 4, 5 or 6; and

B is hydrogen or a group comprising a ligand capable of binding to a specific binding partner;

Z is chloro or bromo;

m is +1 or +2;

X is mono or divalent anion;

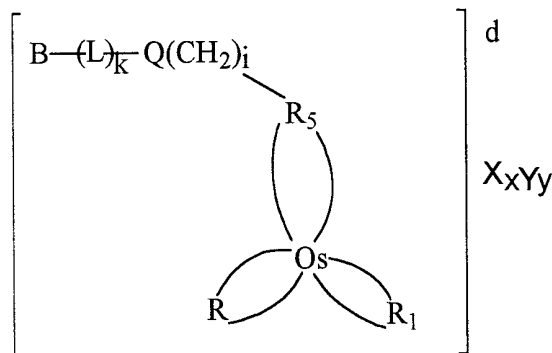
Y is a monovalent anion; and

n is 1 or zero,

provided that when X is a divalent anion, n is zero,

and when m is 1, n is zero and X is not a divalent anion.

21. The device of claim 11 wherein at least one of the redox reversible species is an electrochemically detectable compound of the formula



25 wherein

R and R₁ are the same or different and are 2,2'-bipyridyl, 4,4'-disubstituted-2,2'-bipyridyl, 5-5'-disubstituted,-2,2'-bipyridyl, 1,10-phenanthrolinyl, 4,7-disubstituted-1,10-phenanthrolinyl, or 5,6-disubstituted-1,10-phenanthrolinyl, wherein each substituent is a methyl, ethyl, or phenyl group,

30 R₅ is 4-substituted-2,2'-bipyridyl or 4,4'-disubstituted-2,2'-bipyridyl wherein the substituent is the group B-(L)_k-Q(CH₂)_i- and the 4'-substituent is a methyl, ethyl or phenyl group;

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R, R₁ and R₂ are coordinated to Os through their nitrogen atoms;

Q is O, S, or NR₄ wherein R₄ is hydrogen, methyl or ethyl;

-L- is a divalent linker;

k is 1 or 0;

5 i is 1, 2, 3, 4, 5 or 6;

B is hydrogen or a group comprising a ligand capable of binding to a specific binding partner;

d is +2 or +3;

X and Y are anions selected from monovalent anions and divalent
10 anions sulfate, carbonate or sulfite wherein x and y are independently 0, 1, 2, or 3 so that the net charge of X_xY_y is -2 or -3.

22. The device of claim 11 wherein the redox reversible species have respective redox potentials differing by at least 100 millivolts.

23. The device of claim 11 wherein the redox reversible species have
15 respective redox potentials differing by at least 200 millivolts.

24. The device of claim 11 wherein the device comprises at least two electrode structures, each in the form of microarray electrodes dimensioned to enable diffusible recycling of a diffusible redox reversible species.

25. The device of claim 11 for quantifying a first analyte and a second
20 analyte in a liquid sample, said device comprising two redox reversible species, a first redox reversible species comprising a conjugate of a ligand analog of the first analyte and a second redox reversible species comprising a conjugate of a ligand analog of the second analyte, each of said analyte analog conjugates being capable of binding competitively with its respective analyte to a specific binding partner.

25 26. The device of claim 25 further comprising a binding partner specific for both the first analyte and the redox reversible conjugate of the ligand analog of the first analyte and a binding partner specific for both the second analyte and the redox reversible conjugate of the ligand analog of the second analyte.

27. The device of claim 11 further comprising a bipotentiostat in electrical
30 communication with the conductors for applying a redox-reversible-species-dependent-cathodic potential to one working electrode and a redox-reversible-species-dependent-anodic potential to a second working electrode.

28. The device of claim 27 or quantifying one or more analytes in a liquid sample, said device including first and second redox reversible species, wherein the bipotentiostat is programmable, and it is programmed to apply a first cathodic potential to a first working electrode and a first anodic potential to a second working electrode, said first anodic and cathodic potentials corresponding to those potentials necessary to establish current flow through the sample due to diffusional recycling of the first redox reversible species, and wherein the bipotentiostat is programmed to apply a second cathodic potential to said first working electrode and a second anodic potential to the second working electrode, said second cathodic and anodic potentials corresponding to those potentials necessary to establish current flow through the sample due to diffusional recycling of the second redox reversible species, and means for measuring current flow through the sample at each of the first and second potentials.

29. The device of claim 27 for quantifying one or more analytes in a liquid sample, said device including first and second redox reversible species, and at least first and second electrode structures for contact with the liquid sample in the chamber, each of said electrode structures comprising a microarray of working electrodes, and a switch for changing the electrical communication of the bipotentiostat between the first and second electrode structures.

30. The device of claim 29 wherein the bipotentiostat is programmable, and it is programmed to apply a first cathodic potential to a working electrode of the first electrode structure and a first anodic potential to a second working electrode of the first electrode structure, said first anodic and cathodic potentials corresponding to those necessary to establish current flow through the sample due to diffusional recycling of the first redox reversible species, and wherein the bipotentiostat is programmed to apply a second cathodic potential to a working electrode of the second electrode structure and a second anodic potential to a second electrode of the second electrode structure, said second cathodic and anodic potential corresponding to those potentials necessary to establish current flow through the sample due to diffusional recycling of the second redox reversible species, and means for measuring current flow through the sample at each electrode structure.

31. The device of claim 11 wherein the first and second reversible species each comprise a conjugate of different ligand analogs of one analyte, each of

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said conjugates capable of binding competitively with said analyte to one of two independent specific binding partners for said analyte.

32. The device of claim 11 for quantifying glycosylated hemoglobin wherein at least one of the two redox reversible species comprises a conjugate of the
5 formula

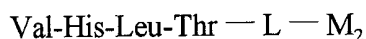


wherein M_1 is a redox reversible label, L is a linker and Gluc-Val-His-Leu-Thr- is the
10 N-terminal sequence of the β -chain of hemoglobin A1c.

33. The device of claim 32 wherein the redox reversible label is a metal ion complex.

34. The device of claim 32 wherein M_1 is an osmium ion complex or ferrocene.

15 35. The device of claim 32 wherein the other reversible redox species comprises a redox reversible conjugate of the formula



20 wherein M_2 is a redox reversible label and L is a linker.

36. The device of claim 35 wherein the redox reversible label is a metal ion complex.

37. The device of claim 35 wherein the redox potential of M_1 and M_2 differ by at least 100 millivolts.

25 38. The device of claim 35 wherein the redox potential of M_1 and M_2 differ by at least 200 millivolts.

39. The device of claim 35 further comprising a specific binding partner for both hemoglobin A1c and the redox reversible conjugate $\text{Gluc-Val-His-Leu-Thr} - \text{L} - \text{M}_1$.

30 40. The device of claim 27 further comprising a specific binding partner for both hemoglobin and the redox reversible conjugate $\text{Val-His-Leu-Thr} - \text{L} - \text{M}_2$.

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41. A kit for measuring the concentration of one or more analytes in a liquid sample, said kit comprising
- at least two redox reversible species for contact with the liquid sample, each capable of diffusion in the liquid sample at least in the presence of a
 - 5 predetermined analyte, at least one species comprising a conjugate of a ligand analog of an analyte and a redox reversible label, said redox reversible species having respective redox potentials differing by at least 50 millivolts;
 - a specific binding partner for each analyte;
 - an electrode structure for contact with the liquid sample, said electrode
 - 10 structure including a reference electrode and working electrodes dimensioned to allow diffusional recycling of diffusible redox reversible species in the sample when a predetermined redox-reversible-species-dependent-cathodic potential is applied to one working electrode and a predetermined redox-reversible-species-dependent-anodic potential is applied to the second working electrode, said diffusional recycling of said
 - 15 species means sufficient to sustain a measurable current through the sample; and
 - conductors communicating with the respective electrodes for applying said anodic potential and said cathodic potential and for carrying the current conducted by the electrodes.
42. The kit of claim 41 wherein the electrode structure comprises
- 20 microarray electrodes selected from the group consisting of arrays of microdiscs, microbands, or microholes.
43. The kit of claim 41 wherein the electrode structure comprises interdigitated microarray electrodes.
44. The kit of claim 41 wherein the redox reversible species are mixed as a
- 25 composition for contact with the liquid sample.
45. The kit of claim 41 wherein the redox reversible label of at least one redox reversible species comprises an osmium complex.
46. The kit of claim 41 wherein the redox reversible species have respective redox potentials differing by at least 100 millivolts.
- 30 47. The kit of claim 41 wherein the redox reversible species have respective redox potentials differing by at least 200 millivolts.

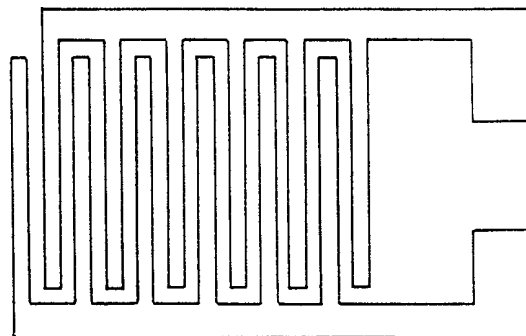
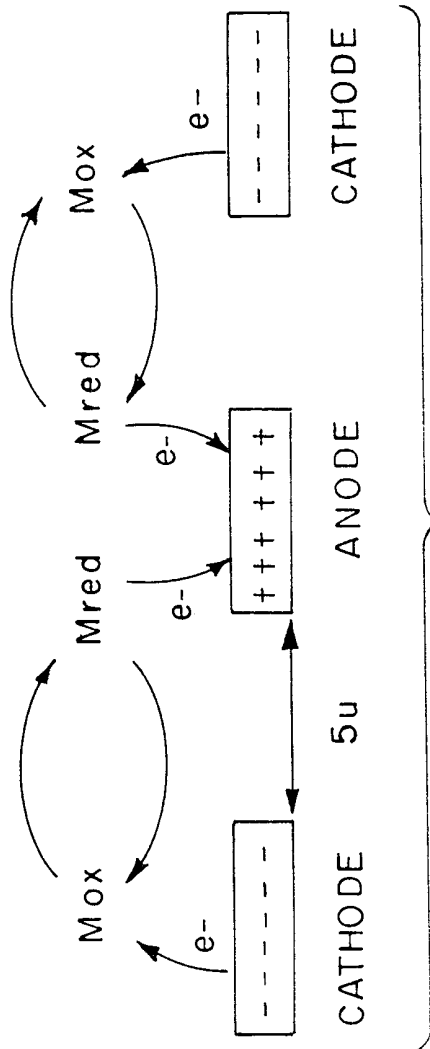


FIG. 1



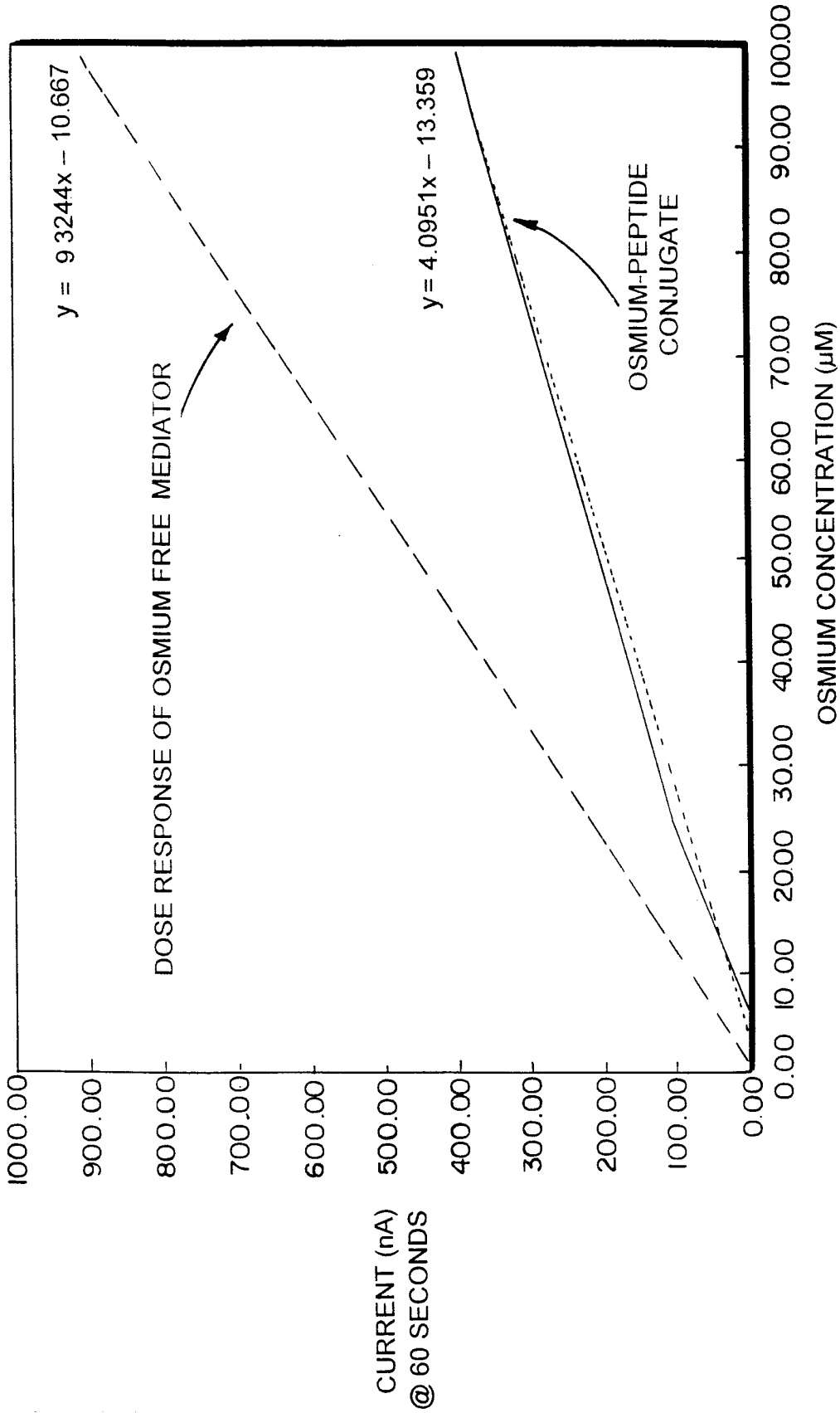
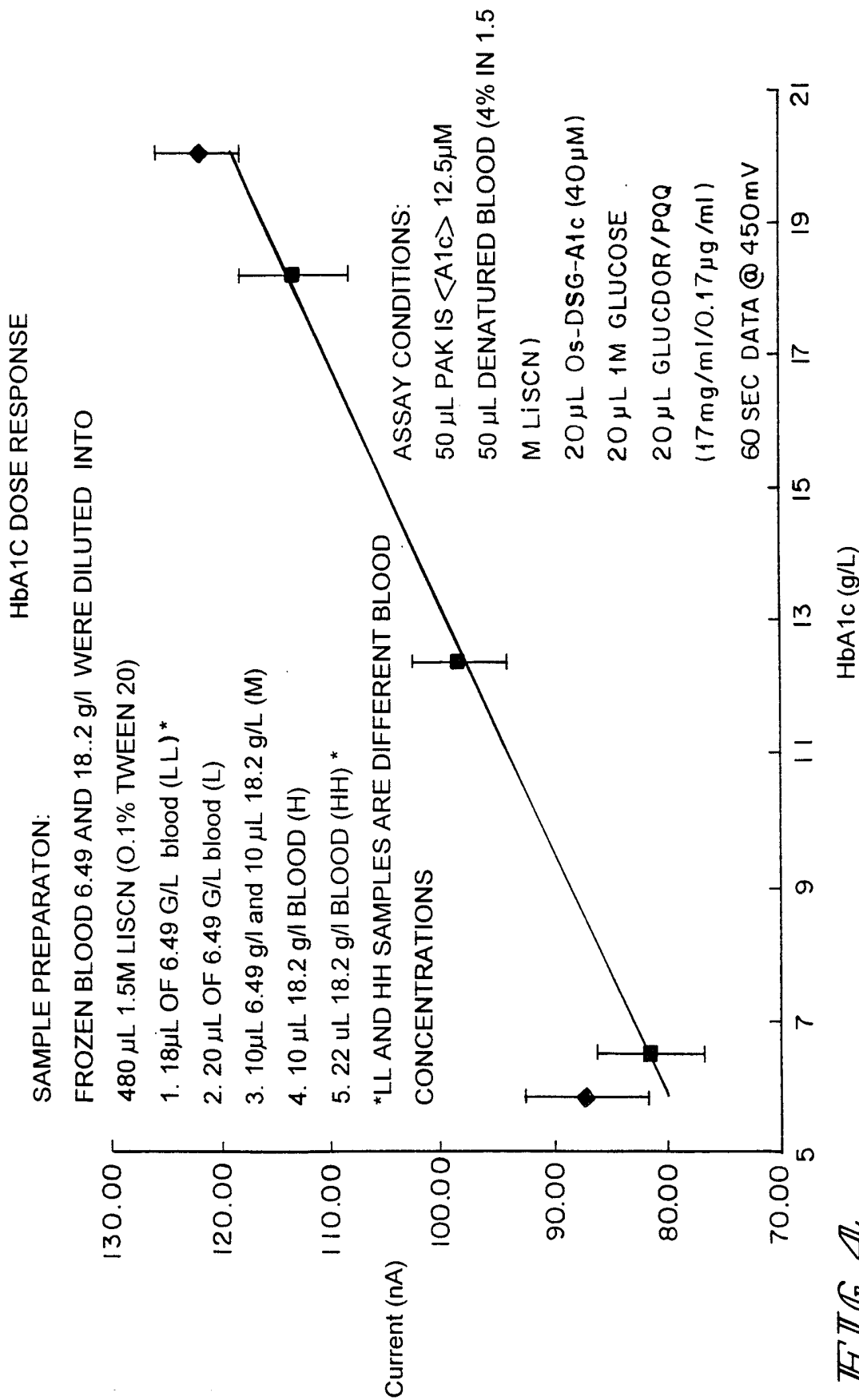


FIG. 3



n=16 for each HbA1c SAMPLE. (4 SAMPLE DENATURATIONS X 4 STRIP REPLICATES)

FIG. 4

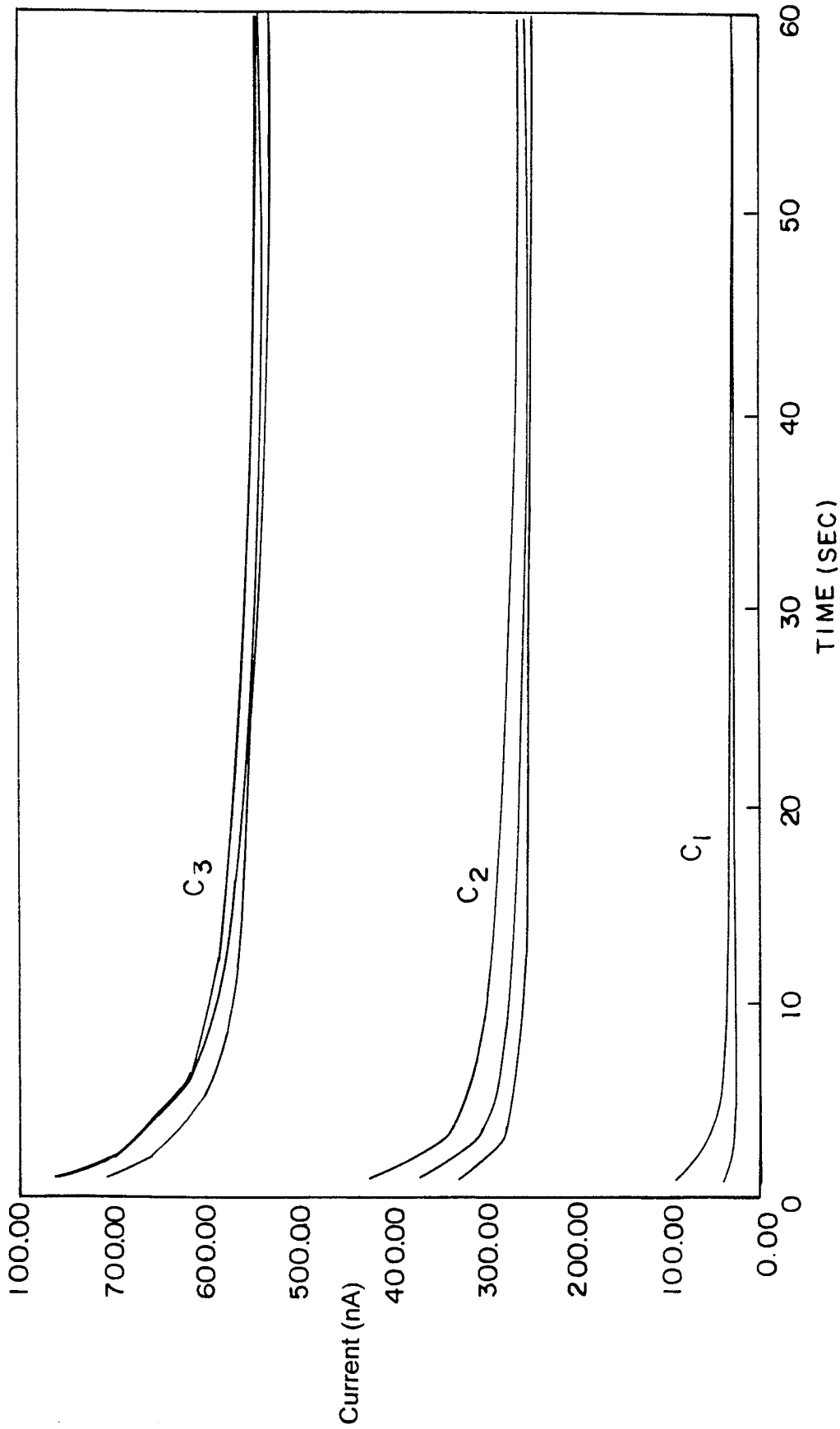


FIG. 5

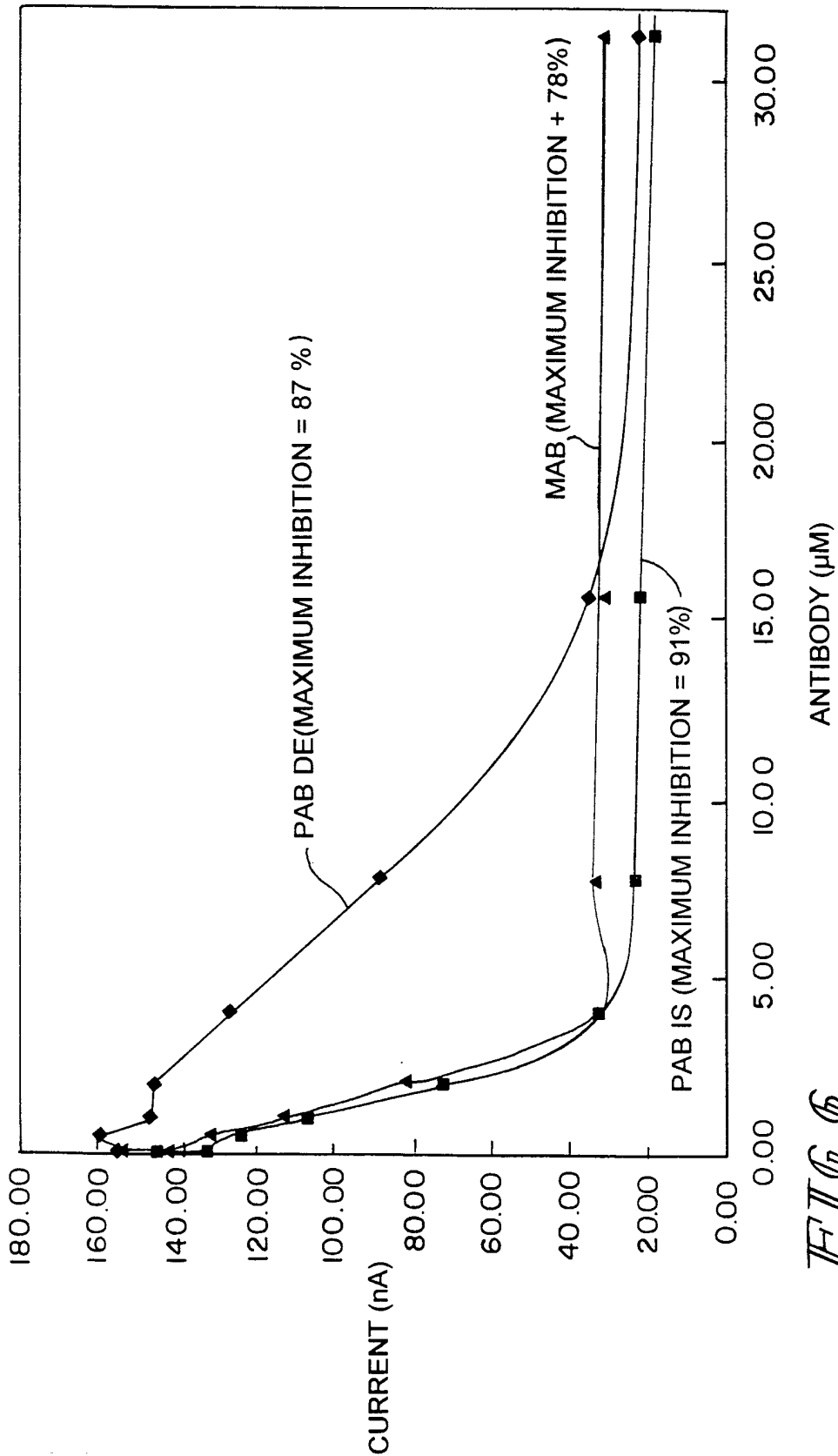


FIG. 6

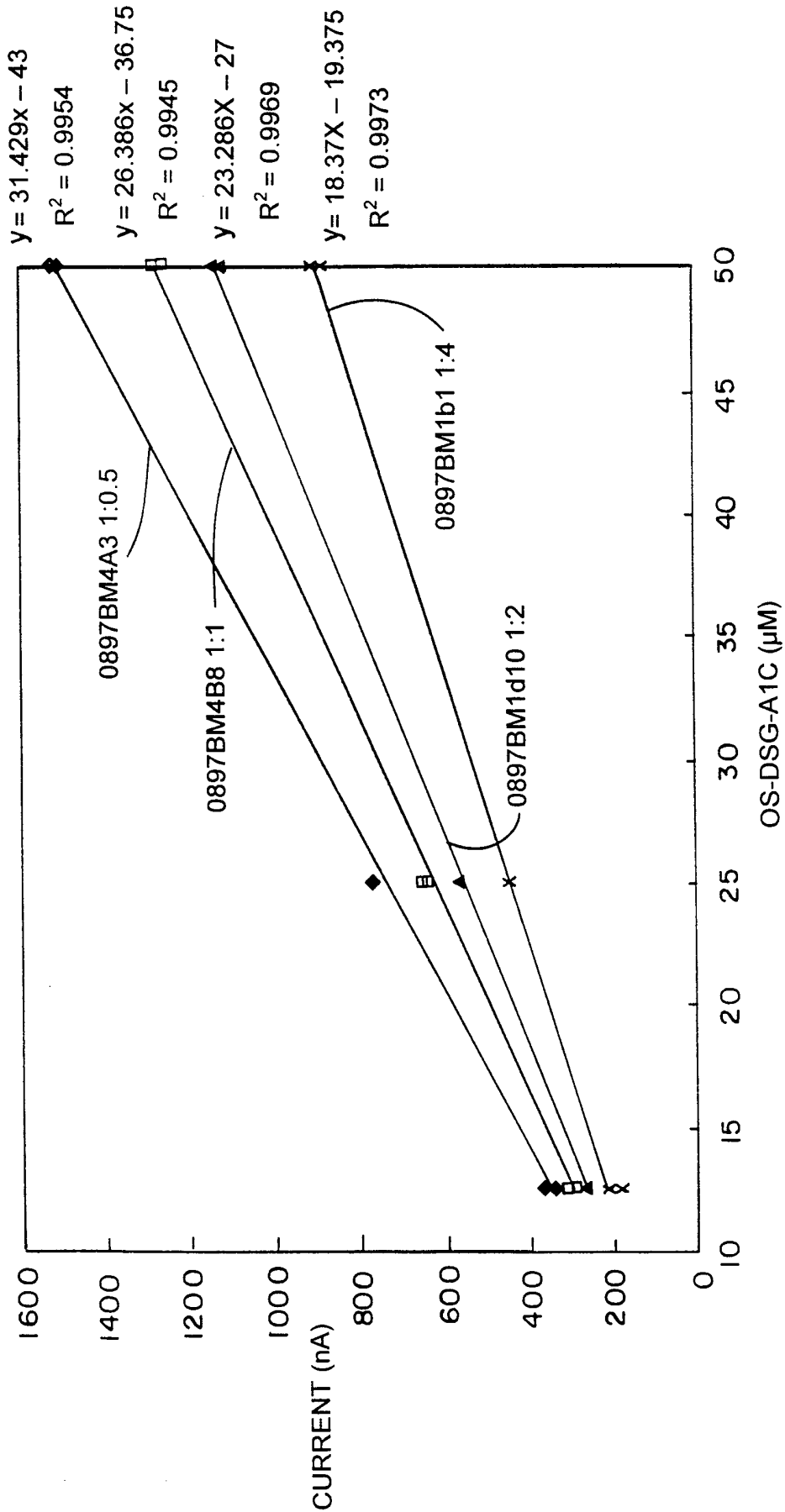


FIG. 7

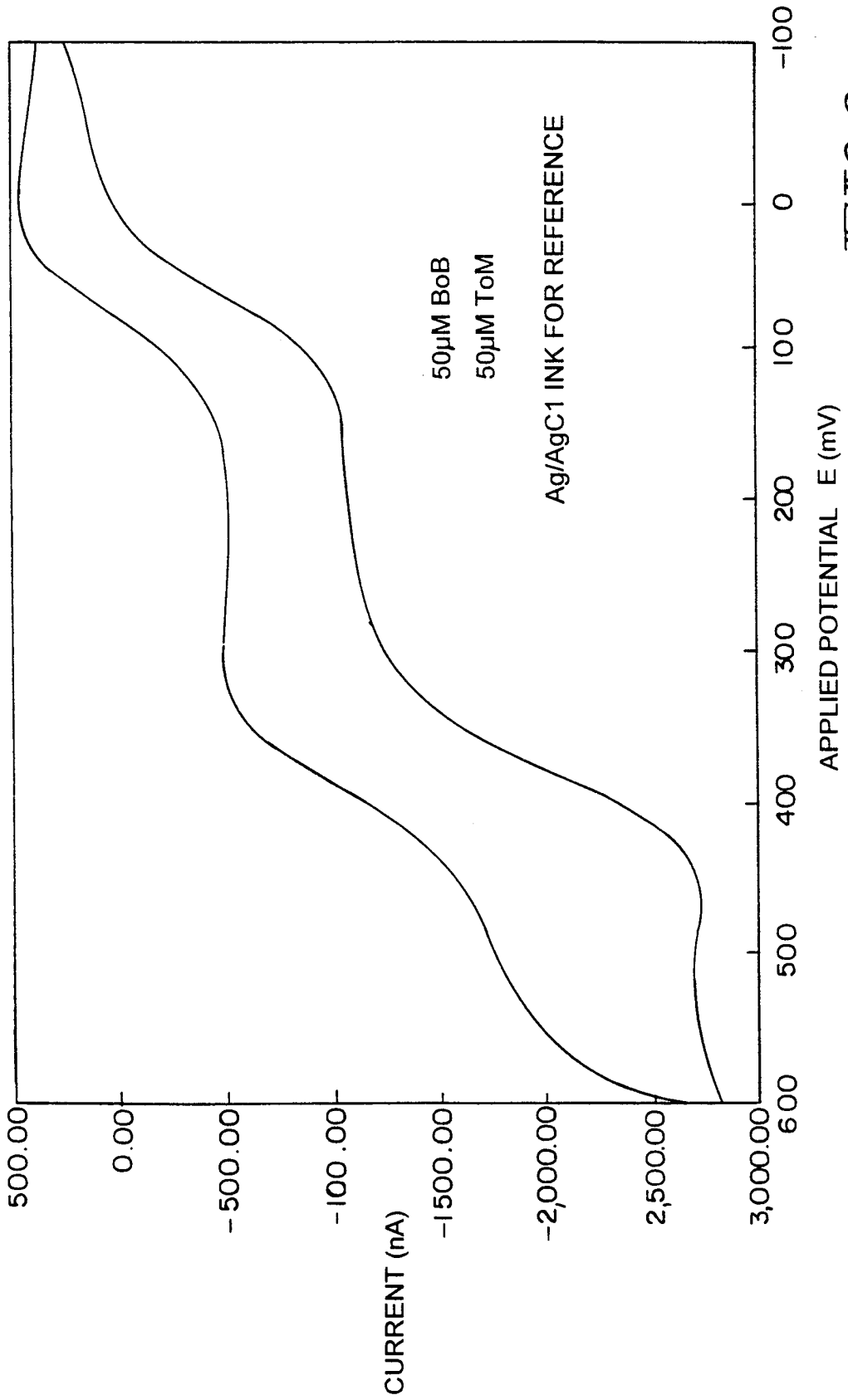


FIG. 8

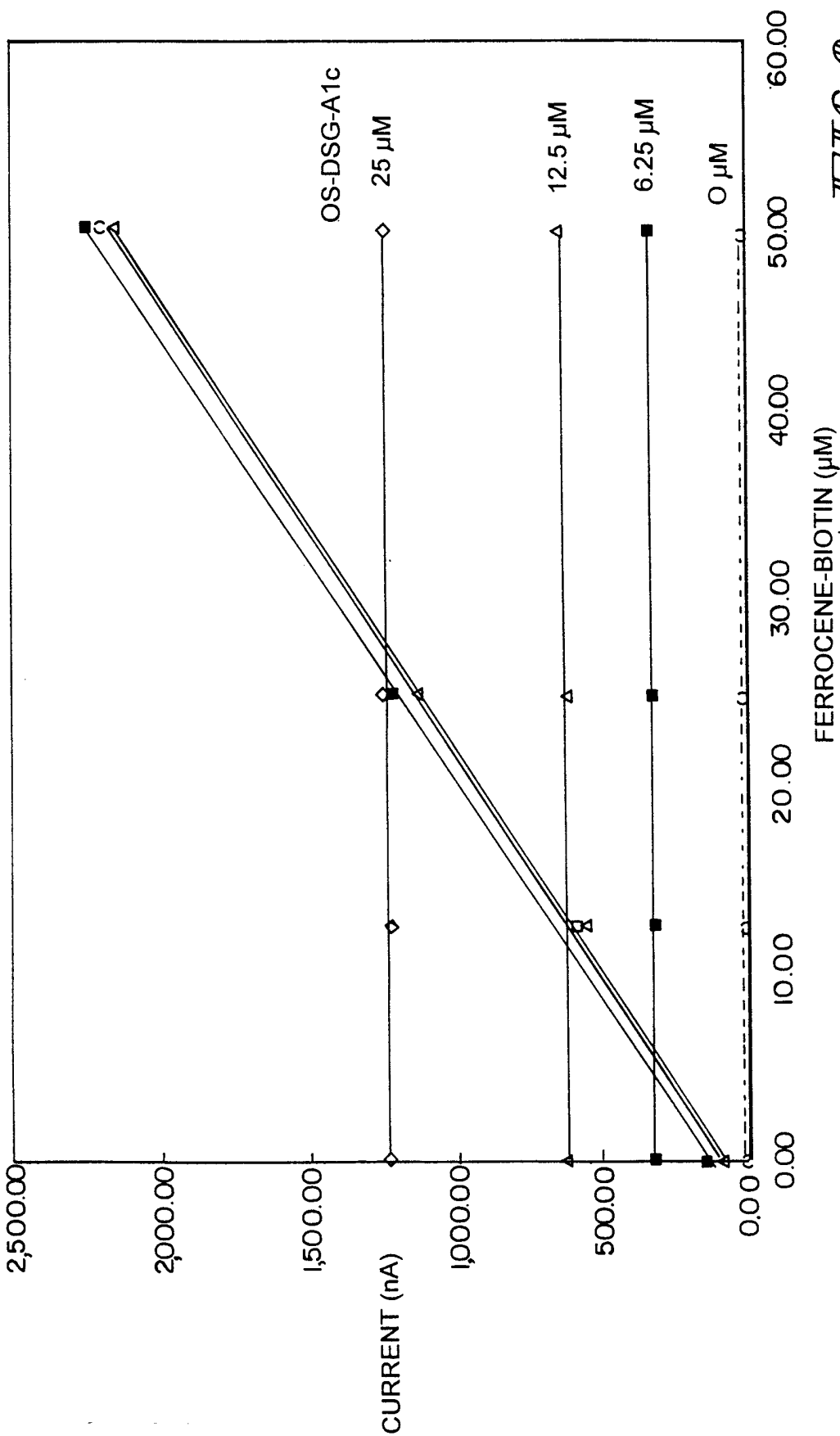


FIG. 9

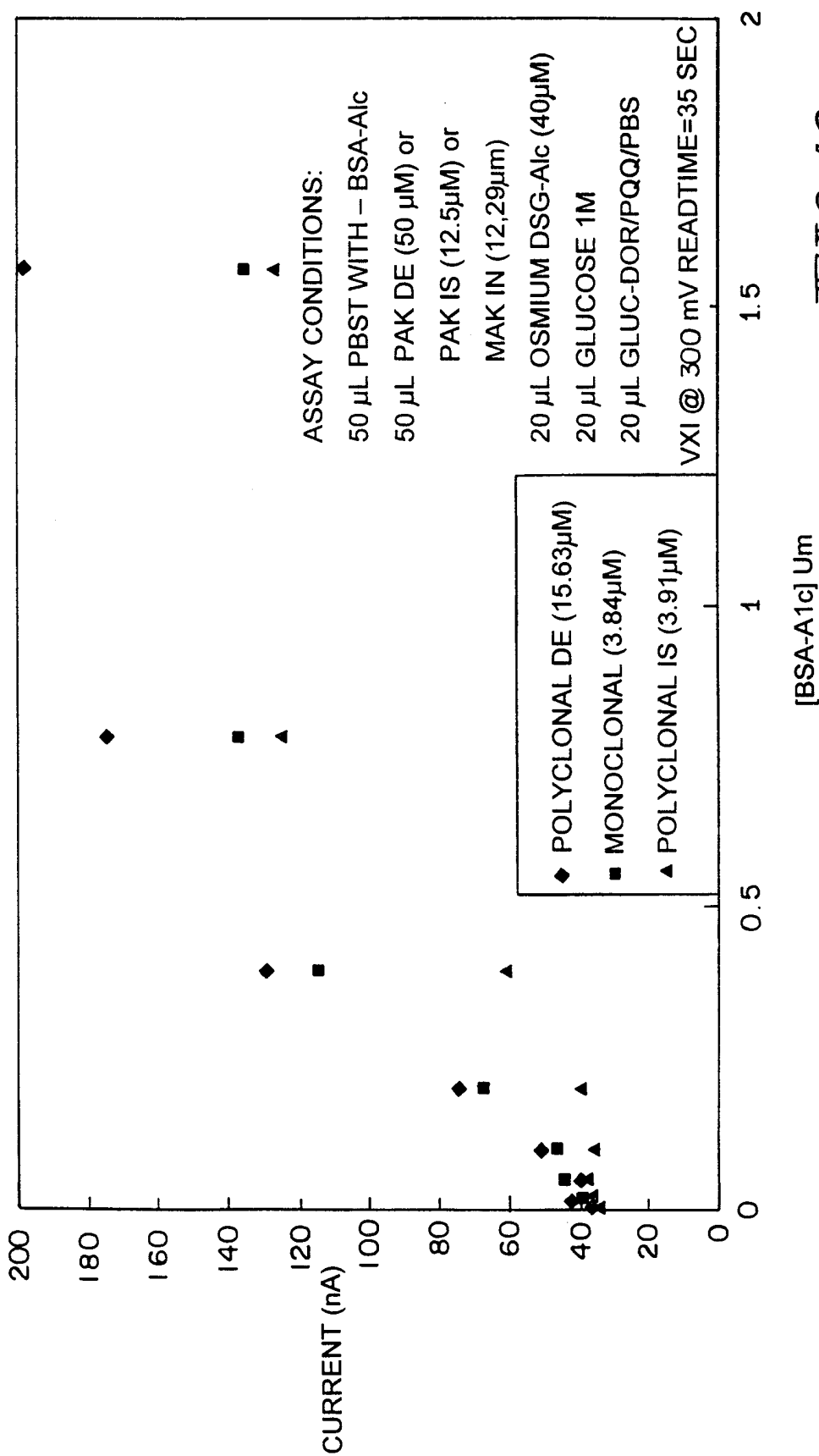


FIG. 10

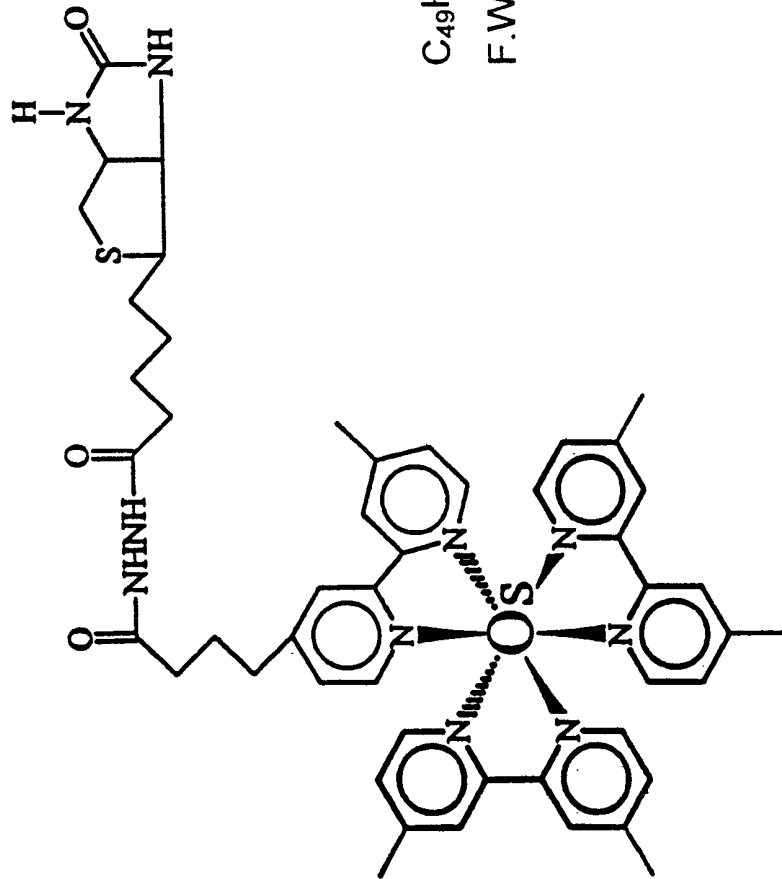
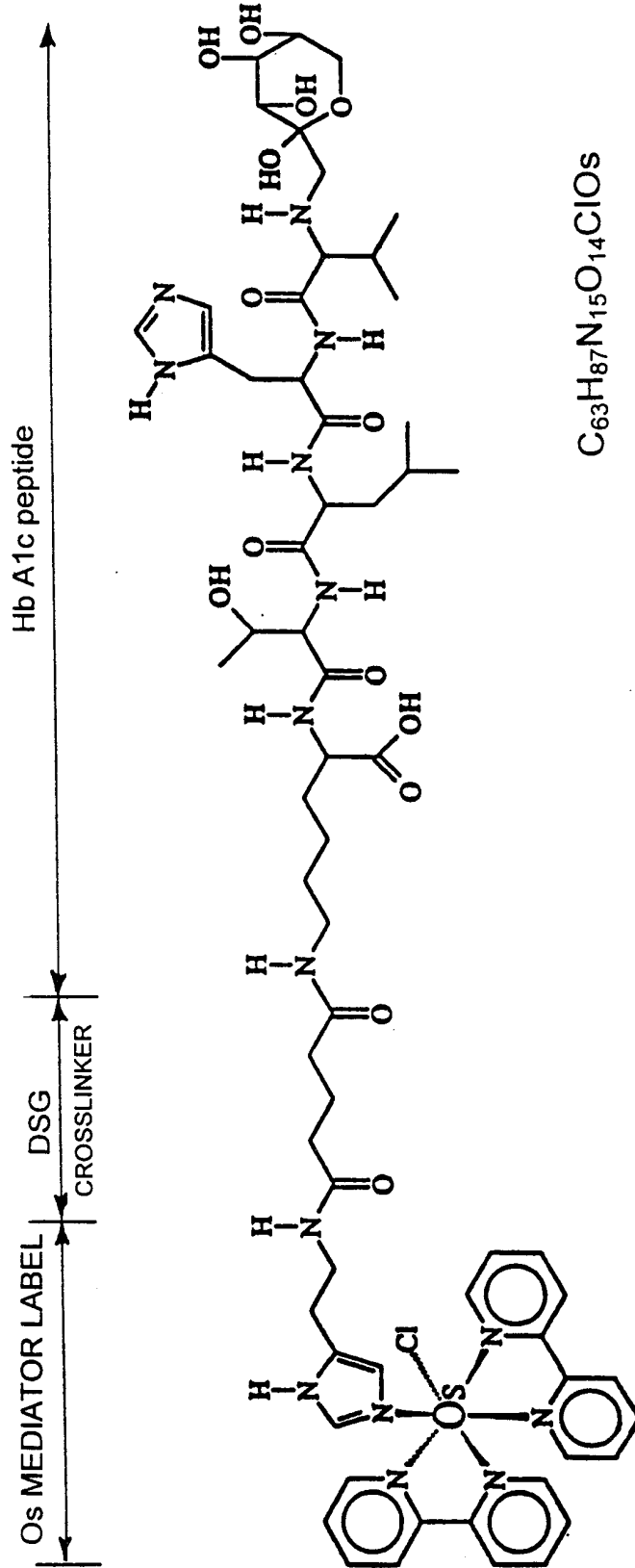


FIG 11



$C_{63}H_{87}N_{15}O_{14}ClOS$

M.W. 1504.12

FIG. 12

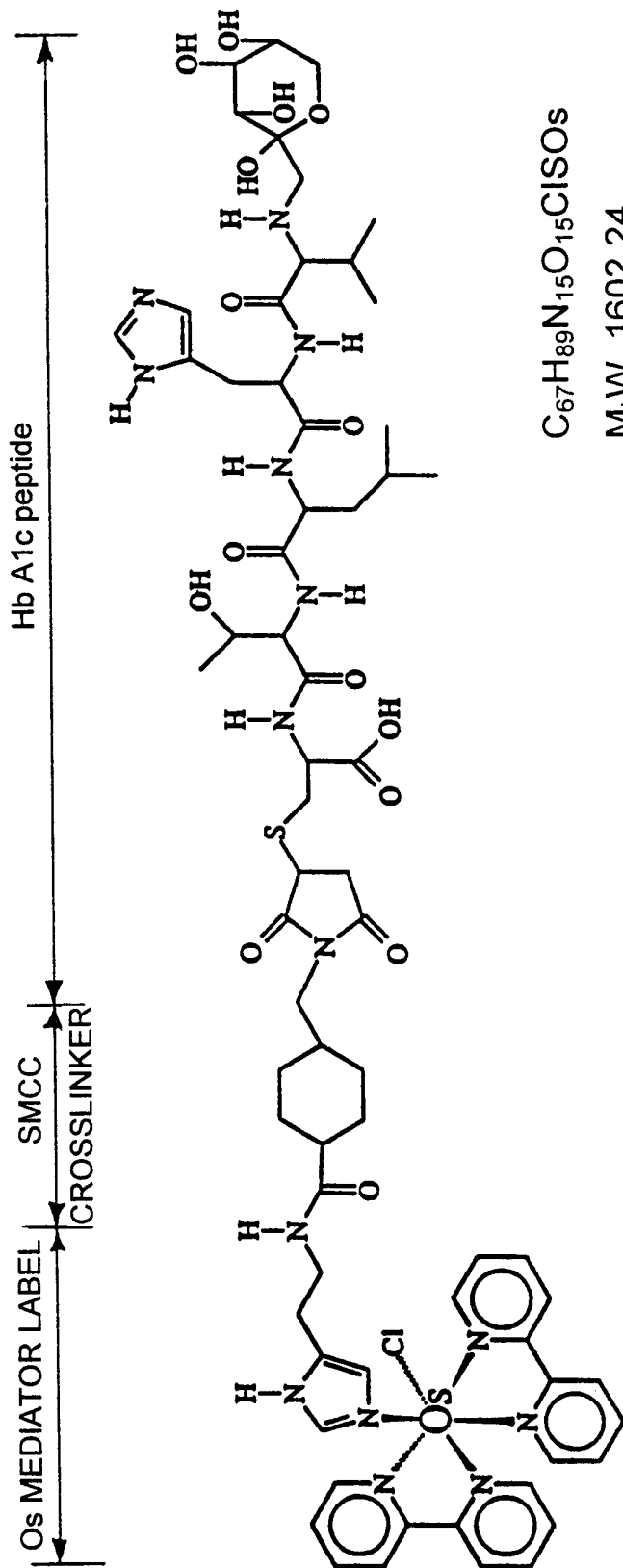
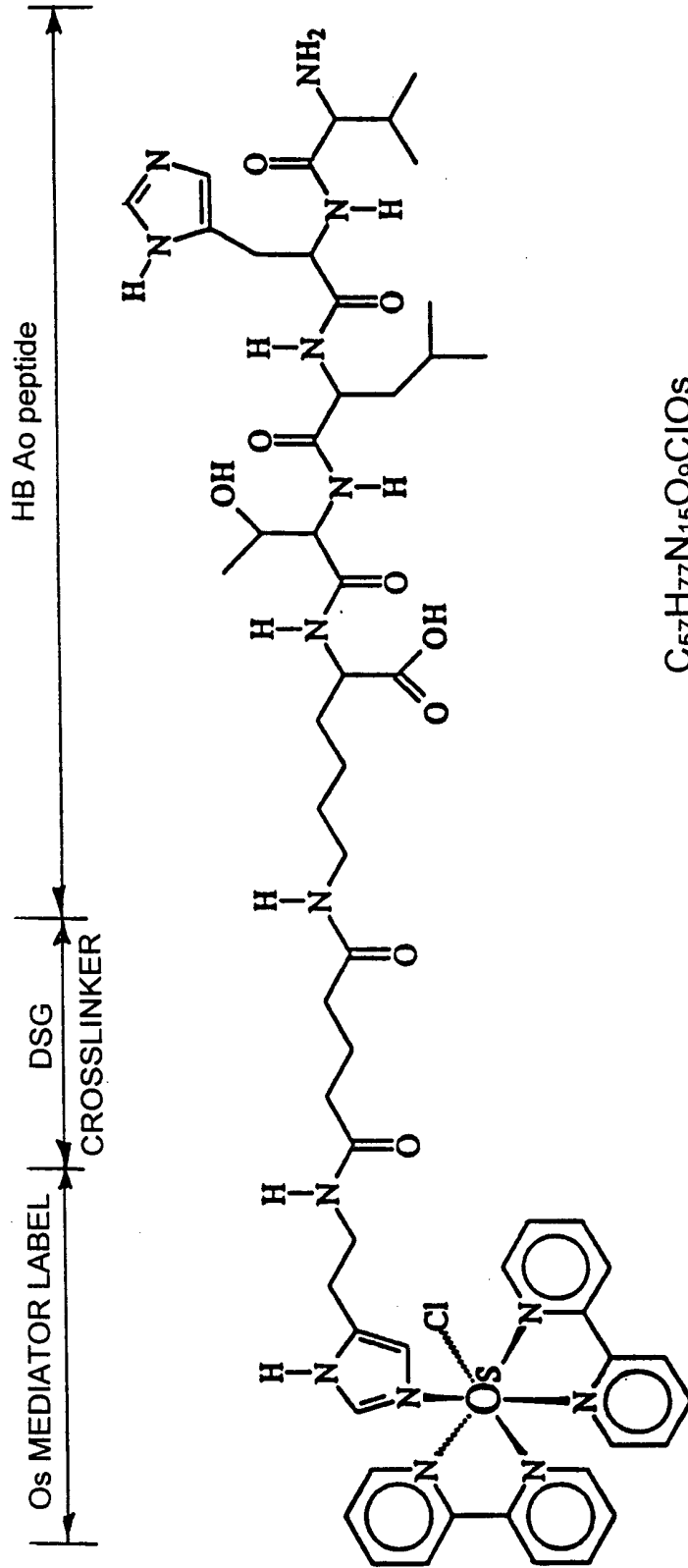


FIG. 13



$C_{57}H_{77}N_{15}O_9ClOs$

M.W. 1341.98

FIG. 14

INTERNATIONAL SEARCH REPORT

International Application No

PC./US 99/11850

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 G01N33/543 G01N33/72

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N C07F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96 41177 A (IGEN INC) 19 December 1996 (1996-12-19) claims 13-17 ---	1-8, 11-15, 17, 22-30, 41-44, 46, 47
Y	US 4 945 045 A (FORREST GORDON C ET AL) 31 July 1990 (1990-07-31) cited in the application example 1 --- -/--	1-8, 11-15, 17, 22-30, 41-44, 46, 47

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

° Special categories of cited documents :

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Date of the actual completion of the international search

10 September 1999

Date of mailing of the international search report

17/09/1999

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Authorized officer

Hart-Davis, J

INTERNATIONAL SEARCH REPORT

International Application No

PC./US 99/11850

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>WO 93 25907 A (CENTRE NAT RECH SCIENT ;DEGRAND CHANTAL (FR); BLANKESPOOR RONALD () 23 December 1993 (1993-12-23)</p> <p>the whole document</p>	<p>1-8, 11-15, 17, 22-30, 41-44, 46,47</p>
A	<p>WO 97 01097 A (SEREX INC) 9 January 1997 (1997-01-09) example 3</p>	<p>11,27, 31-40</p>
A	<p>WO 94 14066 A (UNIV MANCHESTER ;TRELOAR PAUL HOWARD (GB); KANE JOHN WILLIAM (GB);) 23 June 1994 (1994-06-23)</p> <p>page 3, paragraph 4 -page 4, paragraph 2; claims 1-6</p>	<p>1-8, 11-15, 17, 22-30, 41-44, 46,47</p>
A	<p>DE 43 44 646 A (SCHELLER FRIEDER PROF DR) 29 June 1995 (1995-06-29)</p> <p>example 1</p>	<p>1-8, 11-15, 17, 22-30, 41-44, 46,47</p>
A	<p>WO 86 02734 A (HYPERION CATALYSIS INT) 9 May 1986 (1986-05-09) claims 22,24</p>	<p>11,21</p>
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Information on patent family members

International Application No

PCT, US 99/11850

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