

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
10 August 2006 (10.08.2006)

PCT

(10) International Publication Number
WO 2006/084276 A2

(51) International Patent Classification:

C12P 3/00 (2006.01)

(21) International Application Number:

PCT/US2006/004307

(22) International Filing Date: 3 February 2006 (03.02.2006)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

60/649,988 4 February 2005 (04.02.2005) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM,

AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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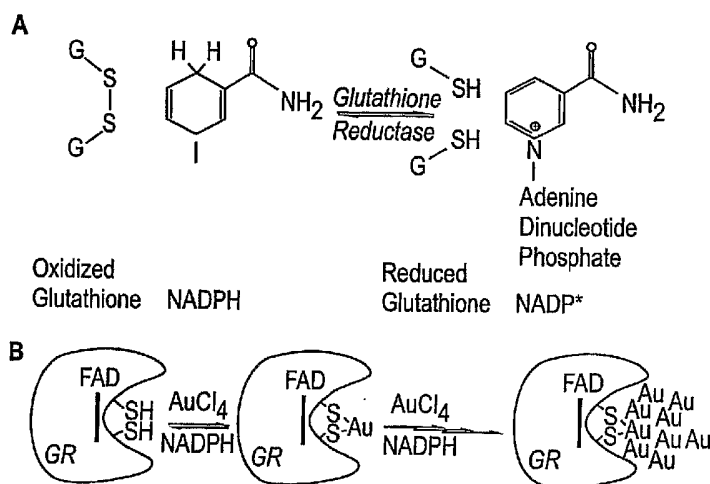
— of inventorship (Rule 4.17(iv))

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ENZYME CATALYZED METALLIC NANOPARTICLE SYNTHESIS



(57) Abstract: The FAD-dependent enzyme glutathione reductase catalyzes the NADPH-dependent synthesis of gold, platinum, and mixed-metal nanoparticles, which are strongly bound to the active site via the redox active cysteine residues. The enzyme stabilizes very small (~5 atom) metallic clusters, and prevents larger clusters from aggregating in the absence of capping ligands. Juxtaposition of the nanoparticle with the FAD cofactor via the active site cysteines enables the maintenance of the nanoparticle at a low potential in the presence of excess NADPH. This allows layered deposition of metals irrespective of redox potential, and the stabilization of low potential metals toward oxidation in aqueous solution. Solid-phase glutathione reductase catalyzed synthesis of gold nanoparticles is demonstrated and proposed as a means to large-scale production of a variety of unique nanoparticle structures.

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Enzyme Catalyzed Metallic Nanoparticle Synthesis

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 60/649,988, filed February 4, 2005.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] NOT APPLICABLE

REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED ON A COMPACT DISK.

[0003] NOT APPLICABLE

BACKGROUND OF THE INVENTION

[0004] Nanotechnology and biotechnology are two fields of ever-increasing importance that are beginning to merge. The former is focused largely on the synthesis and properties of nanometer-scale structures, while the latter largely exploits the extraordinary properties of biological molecules to solve significant medical, chemical, and engineering problems. Applications in which these two fields overlap include quantum dots in optical imaging of biological samples, biosensor applications, and gold nanoparticles in immunochemistry (J. L. West *et al.*, *Annu Rev Biomed Eng*, 5:285 (2003)), as well as the use of gold nanoparticles to facilitate electron transfer between enzymes and electrodes (Y. Xiao *et al.*, *Science*, 299:1877 (Mar 21, 2003)). Thus, biotechnology has historically been the beneficiary of nanotechnology. Here, a role reversal is described in which biotechnology is applied to the furtherance of nanotechnology, with far-reaching implications for nanoscience.

[0005] There is convincing evidence in the literature that biological systems commonly reduce metal ions. For example, bacteria employ mercuric ion reductase in the detoxification of Hg^{2+} , with the product Hg^0 diffusing out of the cell (T. Barkay *et al.*, *FEMS Microbiol Rev*, 27:355 (Jun, 2003)). *Geobacter* species and other chemolithotrophs employ metal ions as terminal electron acceptors (J. R. Lloyd *et al.*, *Adv Appl Microbiol*, 53:85 (2003)), and

magnetotactic bacteria reduce environmental iron to magnetite in the formation of magnetosomes (D. Schuler, *J Mol Microbiol Biotechnol*, 1:79 (Aug, 1999)). Additionally, it has been demonstrated that bacteria, fungi, and plants can facilitate gold nanoparticle (AuNP) synthesis from gold salts (K. Kashefi *et al.*, *Applied & Environmental Microbiology*, 67:3275 (2001); D. R. Lovley, *Annu Rev Microbiol*, 47:263 (1993); B. Nair *et al.*, *Crystal Growth & Design*, 2:293 (2002); S. S. Shankar *et al.*, *Journal of Materials Chemistry*, 13:1822 (2003); P. Mukherjee *et al.*, *Chembiochem*, 3:461 (2002)).

[0006] It is possible that a redox-active enzyme with an active site that can interact strongly with metals ions might be capable of synthesizing metallic nanoparticles. GR is an NADPH-dependent flavoenzyme of known structure (P. R. E. Mittl *et al.*, *Protein Science*, 3:799 (1994)) that normally catalyzes the reduction of oxidized glutathione via a disulfide exchange reaction involving two active site cysteine residues (Cys42 and Cys47; Figure 1A) (P. Rietveld *et al.*, *Biochemistry*, 33:13888 (1994)). The sulfhydryl groups on the catalytic cysteines provide a good binding site for soft metal ions such as Au^{3+} and Pt^{4+} . This would poise them for reduction by NADPH via flavin-mediated electron transfer (analogous to the reaction catalyzed by the homologous enzyme mercuric ion reductase), potentially giving rise to nanoparticle formation in the active site. Surprisingly, this invention meets this and other needs.

BRIEF SUMMARY OF THE INVENTION

[0007] In one embodiment, the present invention provides a method of preparing a nanoparticle, comprising the step of providing at least one redox-active enzyme-coenzyme complex. The method further comprises the step of contacting the enzyme-coenzyme complex with a first metal ion and an electron-donor such that the enzyme-coenzyme complex repetitively catalyzes the reduction of the metal ion thereby preparing the nanoparticle bound to the enzyme-coenzyme complex via repetitive metal ion reduction by the enzyme-coenzyme complex. The method also comprises the step of collecting the nanoparticle.

[0008] In another embodiment, the method of the present invention further comprises the step of repeating the contacting step with a second metal ion.

[0009] In other embodiments, the enzyme-coenzyme complex is a member of the pyridine nucleotide-disulfide oxidoreductase family of enzymes. In some other embodiments, the

enzyme-coenzyme complex is a member selected from the group consisting of thioredoxin reductase, lipoamide dehydrogenase, trypanothione reductase, coenzyme A disulfide reductase, alkyl hydroperoxide reductase, NADH oxidase, NADH peroxidase, mercuric ion reductase and glutathione reductase. In still other embodiments, the enzyme-coenzyme complex is glutathione reductase.

[0010] In a further embodiment, the coenzyme of the enzyme-coenzyme complex is flavin adenine dinucleotide.

[0011] In some embodiments, the first metal ion is a member selected from the group consisting of Au (III), Pt (IV), Co (II), Ni (II), Fe (III), Ag (I) and Cd (II). In another embodiment, the first metal ion is Au (III). In still another embodiment, the first metal ion is Pt(IV).

[0012] In other embodiments, the electron-donor is nicotinamide adenine dinucleotide (phosphate).

[0013] In another embodiment, the collecting step comprises releasing said nanoparticle from said enzyme-coenzyme complex using a sulfhydryl-containing reagent.

[0014] In a further embodiment, the second metal ion is a member selected from the group consisting of Pt (IV), Co (II), Ni (II) and Fe (III).

[0015] In some embodiments, the enzyme-coenzyme complex is attached to a solid support selected from the group consisting of agarose, cyanogen bromide-activated agarose, Sephadex®, cellulose, dextran, starch, chitin, glass, polystyrene, polyacrylamide, polyethyleneglycol, polyethyleneimine, graphite, silica, silica gel and hydroxyapatite (calcium phosphate).

[0016] In another embodiment, the present invention provides an electron transfer system comprising the following elements in electrical connection: an electron donor or acceptor of the present invention, a redox-active enzyme-coenzyme complex of the present invention, a nanoparticle of the present invention, and an electrode, wherein the metal nanoparticle is grown from the enzyme-coenzyme complex via repetitive metal ion reduction by the enzyme-coenzyme complex.

[0017] In some embodiments, the enzyme-coenzyme complex and the nanoparticle elements of the electron transfer system are electrically connected to said electrode via a

nanotube or nanowire. In another embodiment, the nanotube is a single-walled carbon nanotube. In still another embodiment, the nanowire is a LiMo_3Se_3 nanowire.

[0018] In a further embodiment, the present invention provides a method of generating an electrical current using the electron transfer system of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] Figure 1. (A) The cognate reaction catalyzed by GR, reduction of oxidized glutathione. (B) Schematic of nanoparticle synthesis catalyzed by GR.

[0020] Figure 2. Absorbance spectra of FAD bound to GR. The oxidized form of the enzyme is shown in blue, the reduced form obtained by NADPH addition to the oxidized form is shown in red, and the reduced enzyme to which excess AuCl_4^- was added is shown in green. The addition of AuCl_4^- reoxidizes the reduced enzyme, demonstrating electron transfer from NADPH to AuCl_4^- , mediated by the GR-bound FAD. The inset shows the initial rates of oxidation of NADPH or NADH in the presence and absence of AuCl_4^- . The lower rate of NADH oxidation is consistent with the known preference of GR for NADPH (N. S. Scrutton *et al.*, *Nature*, 343:38 (Jan 4, 1990)).

[0021] Figure 3. (A) MALDI-TOF mass spectra of GR reacted with excess NADPH and various stoichiometries of AuCl_4^- . The higher m/z peaks are for the singly charged dimeric forms of the GR holoenzyme with bound gold, while the lower m/z peaks are for the doubly charged forms. The inset shows the linear correlation between the number of gold atoms added per enzyme active site and the number found by mass spectrometry. (B) MALDI-TOF mass spectra of GR reacted first with either AuCl_4^- or PtCl_6^{2-} followed by a second metal salt (nickel (II) acetate, cobalt (II) chloride, iron (III) chloride). A sample of the enzyme was removed for mass spectrometry after reaction with Au or Pt and before the second metal salt was added. The number of Ni, Co, or Fe atoms added was calculated from the mass difference between the samples taken before and after addition of the second metal salt.

[0022] Figure 4. (A) Schematic diagram of the use of resin-immobilized GR to synthesize nanoparticles in a batchwise process. The enzyme was attached to cyanogen bromide activated agarose. NADPH and AuCl_4^- were added, followed by mercaptoethylamine to release the GR-bound AuNPs. (B) Transmission electron micrograph of the AuNPs released from the resin-bound GR by mercaptoethylamine.

[0023] Figure 5. Rate calculation for the GR catalyzed NADPH oxidation in the presence of AuCl_4^- showing saturation kinetics.

[0024] Figure 6. MALDI spectra for a GRPtAu complex.

[0025] Figure 7. Energy dispersive x-ray spectroscopy showing resin-bound metal to be gold.

[0026] Figure 8. Demonstration of current flow increasing as the size of the nanoparticle increases.

[0027] Figure 9. Cyclic voltammetry showing the pH dependence of the redox potential of graphite-bound GR-Au50.

[0028] Figure 10. Redox potential vs. pH data for the same *E. coli* GR in solution, taken from a literature paper (Veine DM, Arscott LD, Williams CH Jr. "Redox potentials for yeast, *Escherichia coli* and human glutathione reductase relative to the NAD^+/NADH redox couple: enzyme forms active in catalysis." *Biochemistry* (1998) 37, 15575-82).

[0029] Figure 11. Plot of the redox potential vs. pH data for graphite-bound GR-Au50.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

[0030] As used herein, the term "nanoparticle" refers to a defined particle of typically 5 to 500 atoms. Typical dimensions of the nanoparticles of the present invention are on the scale of a few nanometers, and can be tens of nanometers. The nanoparticles of the present invention typically have dimensions of less than 100 nanometers.

[0031] As used herein, the term "redox-active enzyme-coenzyme complex" refers to an enzyme-coenzyme complex that is capable of participating in a reduction-oxidation reaction. Enzyme-coenzyme complexes useful in the present invention include those that can be oxidized as well as those that can be reduced. The enzyme-coenzyme complexes of the present invention can be catalytic, and include, but are not limited to, the pyridine nucleotide-disulfide oxidoreductase family of enzymes. Examples of the pyridine nucleotide-disulfide oxidoreductase family of enzymes include, but are not limited to, thioredoxin reductase, lipoamide dehydrogenase, trypanothione reductase, coenzyme A disulfide reductase, alkyl

hydroperoxide reductase, NADH oxidase, NADH peroxidase, mercuric ion reductase and glutathione reductase.

[0032] As used herein, the term “coenzymes” refers to a non-proteinaceous organic or inorganic component that functions by mediating transfer of an electron from the donor to the metal ions thereby reducing the metal ions. In some instances, the coenzymes of the present invention include, but are not limited to, flavin adenine dinucleotide (FAD).

[0033] As used herein, the term “metal ion” refers to elements of the periodic table that are metallic and that are negatively or positively charged as a result of having more or fewer electrons in the valence shell than is present for the neutral metallic element. Metals that are useful in the present invention include the earth metals, alkali earth metals, transition metals and post-transition metals. Alkali metals include Li, Na, K, Rb and Cs. Alkaline earth metals include Be, Mg, Ca, Sr and Ba. Transition metals include Sc, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Y, Zr, Nb, Mo, Tc, Ru, Rh, Pd, Ag, Cd, La, Hf, Ta, W, Re, Os, Ir, Pt, Au, Hg and Ac. Post-transition metals include Al, Ga, In, Tl, Ge, Sn, Pb, Sb, Bi, and Po. One of skill in the art will appreciate that the metals described above can each adopt several different oxidation states, all of which are useful in the present invention. In some instances, the most stable oxidation state is formed, but other oxidation states are useful in the present invention. Metal ions useful in the present invention include, but are not limited to, Au (III), Pt (IV), Co (II), Ni (II), Fe (III), Ag (I) and Cd (II), Pd (II), Pb (II), Ru (IV), Cr(VI), Mn (VII), Zn (II), Os (IV), Ir (IV), Mo (VI), Cu (II) and Rh (III).

[0034] As used herein, the term “electron-donor” refers to a species that is capable of donating an electron to an electron-acceptor in a biological system. In the present invention, “electron-donor” includes, but is not limited to, the reduced form of nicotinamide adenine dinucleotide (phosphate) (NAD(P)H). In other instances, the nicotinamide adenine dinucleotide (phosphate) is in the oxidized form (NAD(P)⁺), operating as an electron-acceptor.

[0035] As used herein, the term “sulfhydryl-containing reagent” refers to a reagent containing a thiol group. Exemplary sulfhydryl-containing reagents include, but are not limited to 2-aminoethanethiol and 2-mercaptoethanol.

[0036] As used herein, the term “collecting” refers to displacing the nanoparticle from the enzyme-coenzyme complex and concentrating the nanoparticles for analysis.

[0037] As used herein, the term “electrically connected” refers to elements that are connected so as to allow the free flow of electrons from one element to another.

[0038] As used herein, the term “nanotube” refers to a nanometer scale cylindrical structure that is hollow in the center and has one or more walls. Exemplary nanotubes useful in the present invention include, but are not limited to, single-walled carbon nanotubes and multi-walled carbon nanotubes. One of skill in the art will appreciate that other nanotubes are useful in the present invention.

[0039] As used herein, the term “nanowire” refers to a nanometer scale wire that can be prepared from a variety of materials including, but not limited to, metals, semiconductors, inorganics, or organic materials. Exemplary materials include, but are not limited to, LiMo_3Se_3 . One of skill in the art will appreciate that other materials are useful as the nanowires of the present invention.

II. Preparation of nanoparticles

A. Redox-active enzyme-coenzyme complex

[0040] Enzyme-coenzyme complexes useful in the present invention include those that can be oxidized as well as those that can be reduced. The enzyme-coenzyme complexes of the present invention can be catalytic, and include, but are not limited to, the pyridine nucleotide-disulfide oxidoreductase family of enzymes. Examples of the pyridine nucleotide-disulfide oxidoreductase family of enzymes include, but are not limited to, thioredoxin reductase, lipoamide dehydrogenase, trypanothione reductase, coenzyme A disulfide reductase, alkyl hydroperoxide reductase, NADH oxidase, NADH peroxidase, mercuric ion reductase and glutathione reductase. In some embodiments, the enzymes include glutathione reductase, thioredoxin reductase, and lipoamide dehydrogenase. In other embodiments, the enzyme is glutathione reductase (GR).

[0041] Coenzymes useful in the present invention include non-proteinaceous organic or inorganic components that functions by mediating transfer of an electron from the donor to the metal ions thereby reducing the metal ions. In some instances, the coenzymes of the present invention include, but are not limited to, flavin adenine dinucleotide (FAD).

B. Metals

[0042] Metals useful in the present invention include elements of the periodic table that are metallic and that are negatively or positively charged as a result of having more or fewer electrons in the valence shell than is present for the neutral metallic element. Metals that are useful in the present invention include the earth metals, alkali earth metals, transition metals and post-transition metals. Alkali metals include Li, Na, K, Rb and Cs. Alkaline earth metals include Be, Mg, Ca, Sr and Ba. Transition metals include Sc, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Y, Zr, Nb, Mo, Tc, Ru, Rh, Pd, Ag, Cd, La, Hf, Ta, W, Re, Os, Ir, Pt, Au, Hg and Ac. Post-transition metals include Al, Ga, In, Tl, Ge, Sn, Pb, Sb, Bi, and Po. One of skill in the art will appreciate that the metals described above can each adopt several different oxidation states, all of which are useful in the present invention.

[0043] In some instances, the most stable oxidation state is formed, but other oxidation states are useful in the present invention. Metal ions useful in the present invention include, but are not limited to, Au (III), Pt (IV), Co (II), Ni (II), Fe (III), Ag (I) and Cd (II), Pd (II), Pb (II), Ru (IV), Cr(VI), Mn (VII), Zn (II), Os (IV), Ir (IV), Mo (VI), Cu (II) and Rh (III).

[0044] In some embodiments, the first metal ion is a member selected from the group consisting of Au, Pt, Co, Ni, Fe, Ag and Cd. In other embodiments, the first metal ion is a member selected from the group consisting of Au (III), Pt (IV), Co (II), Ni (II), Fe (III), Ag (I) and Cd (II). In another embodiment, the first metal ion is Au (III). In still another embodiment, the first metal ion is Pt(IV).

[0045] In some instances, only one metal is useful in the present invention. In other instances, a combination of metals is useful in the present invention. In some embodiments, the second metal ion is a member selected from the group consisting of Au, Pt, Co, Ni, Fe, Ag and Cd. In other embodiments, the second metal ion is a member selected from the group consisting of Pt (IV), Co (II), Ni (II) and Fe (III). Combinations of metals useful in the present invention include, but are not limited to, Au and Pt, Au and Ni, Au and Co, Au and Fe, Pt and Ni, Pt and Co, and Pt and Fe. Combinations of three or more metals are also useful in the present invention.

[0046] Commonly, core-shell structures result from syntheses in which two different metal ions are initially mixed with reductant. This is due to the relative order of the redox potentials, with the metal of higher potential being deposited first, forming the core, followed

by the metal of lower potential depositing to form the shell (M. C. Daniel *et al.*, *Chem Rev*, 104:293 (Jan, 2004)). In the presence of excess NADPH, the maintenance of the nanoparticle at a negative potential in the active site of GR potentially allows one to circumvent metal deposition based strictly on redox ordering. For example, one could initially deposit a metal of lower potential followed by a metal of higher potential, without any associated oxidation of the first metal deposited (M. C. Daniel *et al.*, *Chem Rev*, 104:293 (Jan, 2004), Y. Sun *et al.*, *Science*, 298:2176 (Dec 13, 2002)), since the FAD supplies low potential electrons that are used preferentially in metal reduction.

[0047] The GR catalyzed synthesis of metallic nanoparticles enables the preparation of nanoparticle structures that are inaccessible by solution techniques, due to the asymmetry of the enzyme structure. The catalytic apparatus rests at the bottom of a cavity (~15x17x13 Å) in the protein (P. R. E. Mittl *et al.*, *Protein Science*, 3:799 (1994)). Nanoparticle nucleation happens at the active site cysteines and growth can only occur outward (toward solvent) from this locus due to the steric bulk of the protein matrix. Mixed-metal nanoparticles are preferred to be layered, rather than the symmetric core-shell structures that are commonly formed in solution reactions.

C. Electron donors

[0048] Electron donors useful in the present invention include, but are not limited to, nicotinamide adenine dinucleotide (phosphate). One of skill in the art will appreciate that other electron donors are also useful in the present invention.

D. Solid support

[0049] A variety of solid supports are useful in the present invention including, but not limited to, agarose, cyanogen bromide-activated agarose, Sephadex®, cellulose, dextran, starch, chitin, glass, polystyrene, polyacrylamide, polyethyleneglycol, polyethyleneimine, graphite, silica, silica gel and hydroxyapatite (calcium phosphate). In some embodiments, the solid support is agarose. In other embodiments, the solid support is cyanogen bromide-activated agarose. One of skill in the art will appreciate that other solid support materials are also useful in the present invention.

E. Nanotube and nanowire connections to the electrode

[0050] The nanoparticles of the present invention can be linked to the electrodes of the present invention directly, or via a nanotube or nanowire. Nanotubes useful in the present invention include, but are not limited to, carbon nanotubes. In some instances, single-walled carbon nanotubes (SWCNTs) are useful in the present invention. In other instances, multi-walled carbon nanotubes are useful in the present invention. Nanotubes of other material are also useful.

[0051] Nanowires useful in the present invention can be prepared from a variety of materials including LiMo_3Se_3 . One of skill in the art will appreciate that other materials can be used for the nanowires of the present invention.

F. Collection of the nanoparticles

[0052] The nanoparticles of the present invention can be collected using a variety of techniques known to one of skill in the art. Collection first requires releasing the nanoparticle from the enzyme-coenzyme complex. This can be accomplished by adding a competitive binding agent that displaces the enzyme-coenzyme complex. Following displacement of the enzyme-coenzyme complex, the nanoparticle can then be collected using a variety of techniques known to one of skill in the art, such as filtration or centrifugation.

III. Electron transfer system

[0053] The present invention provides an electron transfer system comprising the following elements in electrical connection: an electron donor or acceptor, a redox-active enzyme-coenzyme complex, a nanoparticle, and an electrode, wherein the metal nanoparticle is grown from the enzyme-coenzyme complex via repetitive metal ion reduction by the enzyme-coenzyme complex. The electron transfer systems of the invention can be used as electrodes (either anode or cathode) in an enzyme-based fuel cell wherein the $\text{NAD(P)}^+/\text{NAD(P)H}$ pair acts as an electron shuttling component. The electron transfer systems can also be used for the purpose of sensitive amperometric detection of compounds that are acted on by NAD(P)^+ dependent dehydrogenases, including but not limited to glucose, lactate, ethanol, amino acids, and the like. Applications of the latter mode of employment include but are not limited to blood glucose monitoring for diabetics, either discontinuously or continuously, depending on the mode of employment. Additionally, it can be used to regenerate NAD(P)H electrochemically, which is frequently used as a reductant in enzyme-catalyzed reactions

employed for stereospecific reductions in commercial organic synthetic applications in the pharmaceutical and other industries.

[0054] In some embodiments, the present invention provides that the enzyme-coenzyme complex and the nanoparticle are electrically connected to the electrode via a nanotube or a nanowire. The nanotube can be made of carbon or other materials. The nanotube can have a single wall or have multiple walls. When the nanotube is carbon, the nanotube can be a single-walled carbon nanotube. The carbon nanotube can also be a multi-walled carbon nanotube.

[0055] The electrodes of the present invention are prepared from materials recognized by one of skill in the art.

[0056] The electron transfer system of the present invention provides that the electron donor or acceptor is nicotinamide adenine dinucleotide phosphate. The enzyme-coenzyme complex is a member of the pyridine nucleotide-disulfide oxidoreductase family of enzymes. The enzyme-coenzyme complex is glutathione reductase. The nanoparticle comprises at least one metal selected from the group consisting of Au, Pt, Co, Ni, Fe, Ag and Cd. The enzyme-coenzyme complex and said nanoparticle are electrically connected to said electrode via a nanotube or nanowire. The nanotube is a single-walled carbon nanotube. The nanowire is a LiMo_3Se_3 nanowire.

[0057] The present invention also provides a method of generating an electrical current using the electron transfer system.

IV. Examples

[0058] **Chemicals.** Preformed 2 nm AuNPs were purchased from Ted Pella, Inc. Nickel (II) acetate, cobalt (II) chloride, iron (III) chloride, silver nitrate, and cadmium (II) acetate were purchased from Fisher Scientific. KH_2PO_4 and triethanolamine (TEA) was purchased from EM Science. Oxidized glutathione (GSSG), reduced nicotinamide adenine dinucleotide phosphate (NADPH), HAuCl_4 , H_2PtCl_6 , and all other chemicals were purchased from Sigma-Aldrich.

[0059] **Instrumentation.** The MALDI mass spectra were acquired on a Perceptive Biosystems Voyager-DE MALDI-TOF and on an AB1 4700 MALDI-TOF.

EXAMPLE 1: PREPARATION OF Au NANOPARTICLES

[0060] Glutathione reductase purification. The gene for *E. coli* glutathione reductase (a gift from Professor Charles Williams) as subcloned into plasmid pProExb-Hta and overexpressed in *E. coli* BL21 DE3 gold. The harvested cells were resuspended in 100 mM potassium phosphate buffer pH 7.6, sonicated at 4 °C for 30 min, and centrifuged at 20,000 g for 40 min. GR was purified from the supernatant using Ni-NTA Superflow resin (Qiagen) via the 6xHis tag added to the GR gene in the pProExb-Hta-GR construct. GR was eluted with 300 mM imidazole, dialyzed into 100 mM potassium phosphate buffer pH 7.6, and stored frozen at -70°C.

[0061] FAD titration with NADPH and AuCl₄⁻. Absorption spectra of GR-bound FAD (100 μM enzyme) were monitored at room temperature in 100 mM potassium phosphate buffer pH 7.6 to determine if electrons from NADPH are passed to AuCl₄⁻ via GR-bound FAD. The 460 and 376 nm peaks of the oxidized form are shifted to 450 and 352 nm in the reduced form generated by the addition of 120 μM NADPH. The addition of 85 μM AuCl₄⁻ causes the FAD peaks to shift back to the oxidized form.

[0062] The ability of AuCl₄⁻ to oxidize the reduced FAD cofactor in GR was first investigated. The spectra presented in Figure 2 show the expected differences between oxidized and reduced GR-bound FAD (P. Rietveld *et al.*, *Biochemistry*, 33:13888 (1994)), the latter obtained by the addition of NADPH (120 μM) to the oxidized enzyme (110 μM). The addition of 85 μM AuCl₄⁻ to the reduced enzyme converts the FAD spectrum back to that of the oxidized enzyme, demonstrating electron transfer from NADPH to AuCl₄⁻ mediated by FAD.

[0063] GR catalyzed reduction of AuCl₄⁻ by NADPH. NADPH (265 μM) oxidation was monitored in the presence of GR (14 μM) and AuCl₄⁻ (72 mM), individually and together, to demonstrate that GR catalyzes the NADPH-dependent reduction of AuCl₄⁻. This was performed in 66 mM potassium phosphate buffer at pH 7.6 at room temperature. The reaction mixtures also contained 0.05 mg/mL glucose oxidase from *Aspergillus niger*, 105 mM glucose and 0.02 mg/mL catalase from bovine liver to remove oxygen from the solution. The sample cuvettes were flushed with Argon.

[0064] The concentration of AuCl₄⁻ was varied from 0.043 mM to 3 mM in the presence of a constant concentration of GR (0.64 μM) and NADPH (253 μM), in 95 mM potassium

phosphate buffer pH 7.6 at room temperature. The initial rates of GR catalyzed NADPH oxidation in the presence of AuCl_4^- showed saturation kinetics (Figure 5). Michaelis-Menten analysis of the data gives $k_{\text{cat}} = 1.2 \text{ s}^{-1}$ and $K_M = 120 \text{ }\mu\text{M}$.

[0065] The steady-state kinetics of GR-catalyzed AuCl_4^- reduction were examined and the results are presented in the inset to Figure 2. In the absence of AuCl_4^- , the 340 nm absorbance from NADPH (265 μM) is stable in a solution containing GR (14 μM) on the minute time scale. The addition of AuCl_4^- (72 μM) leads to the oxidation of NADPH as demonstrated by the decrease in 340 nm absorbance with time. Control experiments without enzyme show this oxidation to be GR catalyzed. The replacement of NADPH with NADH gives a lower rate of cofactor oxidation, in accord with the known preference of GR for NADPH (N. S. Scrutton *et al.*, *Nature*, 343:38 (Jan 4, 1990)). This additionally supports the enzyme catalyzed nature of the AuCl_4^- reduction since NADPH and NADH have nearly identical redox potentials (C. Walsh, *Enzymatic reaction mechanisms* (W. H. Freeman, San Francisco, 1979), pp. xv, 978 p.). The weaker discrimination between NADPH and NADH in AuCl_4^- reduction compared to oxidized glutathione reduction (N. S. Scrutton *et al.*, *Nature*, 343:38 (Jan 4, 1990)) suggests that the rate-limiting half-reaction is the noncognate oxidative one in which AuCl_4^- accepts electrons. The dependence of the initial rate of NADPH oxidation on AuCl_4^- concentration shows saturation kinetics. Michaelis-Menten analysis of the initial rate data yield $k_{\text{cat}} = 1.2 \text{ s}^{-1}$ for the reduction of gold (Figure 5), compared to a value of 600 s^{-1} reported for the reduction of oxidized glutathione (M. P. Deonarain *et al.*, *Biochemistry*, 28:9602 (Dec 12, 1989)) and a value of 18 s^{-1} (S. Engst *et al.*, *Biochemistry*, 37:11496 (Aug 18, 1998)) for the mercuric ion reductase catalyzed reduction of Hg^{2+} , additionally supporting the conclusion that gold reduction is rate-limiting.

[0066] **MALDI Mass Spectroscopy of GR-AuNP.** For a typical experiment, 55 μM GR was incubated for 10 min with 4.5 mM NADPH and 0.28 mM AuCl_4^- in 50 mM potassium phosphate buffer pH 7.6 at room temperature. The solution was passed over a Sephadex G50 gel filtration spin column equilibrated with either water or 0.1 – 1 mM potassium phosphate buffer pH 7.6. The sample was precipitated onto a MALDI target with sinapinic acid in 65% acetonitrile and 1% trifluoroacetic acid.

[0067] Figure 3A presents the MALDI-TOF spectra of GR that was incubated for 30 min in the presence of 4.5 mM NADPH and various stoichiometries of AuCl_4^- . The spectrum of the

enzyme without added AuCl_4^- yields two peaks, one for the singly charged species at $m/z = 105,600$ and one for the doubly charged species at $m/z = 52,800$. These correspond to the dimeric native enzyme with FAD bound. The addition of 20 molecules of AuCl_4^- per enzyme in the presence of excess NADPH produces a spectrum ("GRAu₂₀" in Figure 3A) that is shifted by the expected mass, assuming all 20 atoms of gold are retained on the enzyme in a metallic cluster. Similar mass spectra are presented for stoichiometries of 30 and 50. The series of mass spectra show that the addition of more gold alters the ratio between singly and doubly charged species in favor of the former. The inset to Figure 3A shows a highly linear correlation between the number of AuCl_4^- molecules added per enzyme and the number of gold atoms found per enzyme by mass spectrometry. This linear correlation extends down to 5 atoms of gold per enzyme, demonstrating a remarkable stabilization of tiny gold clusters by GR.

EXAMPLE 2: PREPARATION OF A BIMETALLIC PARTICLE

[0068] Both Pt and Au were found to remain on the enzyme through these manipulations while other metals (*e.g.*, Ag, Cd, and Fe) were not observed. NADPH oxidation in the presence of GR and Ag, Cd, Fe, Co, or Ni salts was not observed. Experiments with metal ions that are incompatible with potassium phosphate buffer were performed in triethanolamine-HCl buffer pH 7.6. Conversely, NADPH oxidation was observed with these metal ions when their addition was initiated with GR to which either Au or Pt was previously added.

[0069] Au and Ni/Co. An example of a synthesis of a bimetallic particle follows. Initially, 100 μM GRAu₁₃ in 90 mM potassium phosphate buffer pH 7.6 was made as described above and exchanged into 100 mM TEA-HCl pH 7.6 by gel filtration. Next, 40 μM GRAu₁₃ was reacted with 4.2 mM NADPH and 5.6 mM of either nickel (II) acetate or cobalt (II) chloride for 2 hours at room temperature. These samples were transferred into water by gel filtration and precipitated for MALDI. The mass increase over the original GRAu₁₃ indicated that the final products were GRAu₁₃Ni₂₅, and GRAu₁₃Co₂₀.

[0070] Au and Pt. Figure 6 shows the MALDI spectra for a GRPtAu complex, produced as follows. First, 46 μM GR and 4.6 mM NADPH were mixed with 2.5 mM PtCl_6^{2-} in 46 mM potassium phosphate pH 7.6 at room temperature, and allowed to incubate for 1 hour. Previous results predicted these conditions to generate GRPt₁₀. The observed mass increase was equivalent to 11 atoms of platinum. The remainder of the sample was placed over a gel

filtration column equilibrated in 100 mM potassium phosphate pH 7.6 to remove unreacted platinum. The sample of purified GRPt₁₁ (~25 μ M) was then reacted with 4.1 mM NADPH and 0.84 mM AuCl₄⁻ in 56 mM potassium phosphate pH 7.6 for 10 min. The mass spectra show that this sample increased by a mass that corresponds to 48 atoms of gold (Figure 6).

EXAMPLE 3: INHIBITION AND REACTIVATION OF GR

[0071] GR activity assays were performed in 10 - 100 mM potassium phosphate buffer pH 7.6, 0.76 nM GR, 0.25 mM NADPH, and 5.0 mM GSSG at room temperature. NADPH oxidation was followed at 340 nm.

[0072] A 0.2 μ M solution of preformed 2 nm AuNPs was incubated with different samples of 0.1 μ M GR. Aliquots of the incubations were tested for their ability to reduce oxidized glutathione (GSSG) in the presence of NADPH. GSSG reduction was inhibited 35% after GR was incubated for 10 min with preformed 2 nm AuNPs in the absence of NADPH. When the incubation was performed in the presence of 0.9 mM NADPH, GR lost >98% of its enzymatic activity after 10 min.

[0073] All GRAu, samples were inactive in the reduction of oxidized glutathione. When, for example, 6.6 μ M GRAu₅₀ in 20 mM potassium phosphate buffer pH 7.6 was reacted with 10 mM 2-aminoethanethiol or 2-mercaptoethanol at room temperature for ~5 min full glutathione reductase activity returned.

[0074] The AuNPs retained on GR are expected to be located in the active site since the sulfhydryl groups of the two catalytic cysteines should bind them strongly. The following lines of evidence demonstrate that they are indeed bound at the active site, which lies at the bottom of the ~15 Å deep cleft observed by X-ray crystallography (P. R. E. Mittl *et al.*, *Protein Science*, 3:799 (1994)). Preformed 2 nm AuNPs added in a 1:1 stoichiometry with enzyme (0.1 μ M) cause loss of activity (>98% after 10 min) in the GR catalyzed reduction of oxidized glutathione. Inhibition occurs >10-fold more rapidly in the presence vs. absence of NADPH in a solution of GR (0.1 μ M) and excess (0.2 μ M) nanoparticles. Faster inhibition by the preformed AuNPs with reduced vs. oxidized GR is expected only if the active site cysteines are the primary site of attachment. The incubation of GR with NADPH and AuCl₄⁻, with consequent AuNP formation, also leads to complete inhibition of GR catalyzed reduction of oxidized glutathione. This activity is regained over the course of ~5 min only when either mercaptoethanol or mercaptoethylamine (10 mM) is added. These small thiol

reagents adsorb to the AuNP surface in the active site, releasing it from the enzyme by thiol exchange with the active site cysteines.

[0075] Finally, the presence of GR protects the AuNPs from aggregation in the absence of exogenous surface ligands (*e.g.* amines or thiols). For example, GRAu₁₀₀ without added surface ligand is stable toward aggregation (as detected colorimetrically by surface plasmon resonance) for hours in buffer at room temperature, with aggregation detected colorimetrically only after prolonged incubation. Inhibition of AuNP aggregation is strong evidence that it is sequestered in the deep active site cleft (as opposed to being on a surface binding site), such that collisional encounter with other AuNPs is prevented by the steric bulk of the enzyme. Taken together, these results convincingly demonstrate that the AuNPs are bound to GR via the two catalytic cysteines deep in the active site cleft, that the nanoparticles readily accept electrons from NADPH via the FAD cofactor, and that they transfer these electrons to metal ions in solution in the course of AuNP growth.

[0076] The apposition of the gold cluster with the FAD in the GR active site, enforced by the location of the catalytic cysteines, creates a unique environment in which the nanoparticle is maintained at a negative potential by reduced FAD in the presence of excess NADPH. This has important implications for the synthesis of mixed-metal nanoparticles. Figure 3B presents mass spectra for GR on which either platinum or gold was initially deposited, followed by metals (Ni, Co, or Fe) of lower redox potential. In the aqueous, oxygen-saturated neutral solutions used here, the metallic state of these low potential metals would be unstable toward oxidation were it not for the presence of excess NADPH acting through FAD in the active site.

EXAMPLE 4: SOLID-PHASE NANOPARTICLE SYNTHESIS

[0077] GR was covalently attached to cyanogen bromide-activated agarose according to the supplier's instructions. GR concentrations on the resin were determined by activity assays with GSSG. The preparation of Au₂₀₀ for imaging was accomplished as follows. GR (50 μ L of CB resin containing 230 nmoles of enzyme in 50 mM phosphate buffer pH 7.6) was allowed to reduce 46 μ moles of AuCl₄⁻ in the presence of NADPH. The concentrations of AuCl₄⁻ and NADPH were maintained at or below 1.1 mM and 2.0 mM, respectively, by adding aliquots of stock solutions in 10 min intervals. Thus, the 230 nmols of GR was allowed to reduce 11.5 μ moles of AuCl₄⁻ every 10 min. The resin was washed with buffer

and then soaked in 100 mM 2-aminoethanethiol for 2 hours. The supernatant was filtered from the resin and analyzed by TEM.

[0078] TEM of a sample of resin soaked in 2-aminoethanethiol showed that a fraction of the reduced Au is not eluted from the agarose resin. Energy dispersive x-ray spectroscopy shows this resin-bound metal to be gold (Figure 7).

[0079] The findings presented here have wide-ranging practical implications if GR can be used in large-scale synthesis of various types of nanoparticles. The experiment illustrated schematically in Figure 4A demonstrates that large-scale GR-catalyzed nanoparticle synthesis can be achieved. GR was attached to cyanogen bromide-activated agarose. NADPH and AuCl_4^- were incubated with the resin to allow nanoparticle synthesis, followed by addition of mercaptoethylamine to release the nanoparticles from the resin-bound enzyme. The effluent from the mercaptoethylamine wash of the resin was analyzed by TEM, and the results are presented in Figure 4B.

[0080] The electron micrograph confirms the presence of individual gold nanoparticles of ~ 2 nm size (in good agreement with the expected 200 atoms of gold per enzyme in this experiment) that were released from the enzyme. The production of metallic nanoparticles of defined size and composition in large quantities is therefore a matter of scaling up this aqueous, green chemistry solid phase methodology, with large-scale batch nanoparticle growth and elution a potentially highly automatable process.

EXAMPLE 5: GENERATION OF AN ELECTRIC CURRENT

[0081] Cyclic voltammetry measurements were performed with a Perkin Elmer 263A Potentiostat using an EG&G micro-cell kit model K0264. Working electrodes were 1 x 1.5 cm Toray graphite TGP-060 donated by Ballard Power Systems.

[0082] The GRAu_{50} complex was examined by cyclic voltammetry (CV) to determine if electron transfer between GR-bound FAD and a graphite electrode is facilitated by the presence of the AuNP. This was done by allowing a 244 μL sample of 50 μM GR, 4.6 mM NADPH, 56 mM potassium phosphate buffer pH 7.6 and either 0.26 mM or 2.6 mM AuCl_4^- to soak at room temperature for 1 hour onto a 1 x 1.5 cm Toray graphite electrode. Electrodes were then rinsed thoroughly before any CV work was done. A gold clip held the electrodes into solution. Measurements were done in 100 mM potassium phosphate buffer pH 7.6 using a saturated calomel electrode as a reference. Data are reported versus the

standard hydrogen electrode (SHE). Platinum wire was used as a counter electrode. Argon gas was used to deoxygenate the cell. The scan rate for the CV measurements in Figure 8 was 100 mV/sec.

[0083] It was found that the AuNP in the active site significantly facilitated the transfer of electrons between the electrode and the FAD in the GRAuNP complex. As shown in Figure 8, under similar experimental conditions current flow increases as the size of the nanoparticle increases. The potential observed in the cyclic voltammetry with GRAu₅₀ (~280 mV) agrees well with that observed for GR-bound FAD in the literature (Figure 5). A control containing no NADPH is presented as the unmodified sample in Figure 8.

Supplementary Information on Carbon Electrode Bound GR-Au50

[0084] The previous data supplied as Figure 8 are evidence that the addition of a AuNP to GR enhances electrical communication between the enzyme and a graphite electrode. In an effort to provide further evidence that the observed oxidation and reduction peaks in the cyclic voltammetry experiment are indeed those for the enzyme-bound FAD, the pH dependence of the redox potential of graphite-bound GR-Au50 was examined in cyclic voltammetry experiments.

[0085] The primary data are presented here in Figure 9 below. One sees a systematic shift of the redox potential to more negative values as the pH is increased. Figure 10 presents redox potential vs. pH data for the same *E. coli* GR in solution, taken from a literature paper (Veine DM, Arscott LD, Williams CH Jr. "Redox potentials for yeast, Escherichia coli and human glutathione reductase relative to the NAD⁺/NADH redox couple: enzyme forms active in catalysis." *Biochemistry* (1998) 37, 15575-82). Figure 11 is a plot of the redox potential vs. pH data for graphite-bound GR-Au50 presented in Figure 9. This plot shows an extremely high degree of similarity to that for the free enzyme in solution (Figure 10). This demonstrates that the observed enhancement of the oxidation and reduction peaks in the cyclic voltammetry experiments is due to the enhancement of electrical communication between the enzyme-bound FAD and the graphite electrode due to the presence of the AuNP in the active site of GR. It also demonstrates that the presence of a AuNP in the active site of GR does not significantly alter the redox potential of FAD in the active site.

EXAMPLE 6: CONNECTING THE GR-AuNP TO THE ELECTRODE

[0086] **Single-wall carbon nanotubes.** Another method for electrically connecting GR-AuNP to graphite is to use a single-walled carbon nanotube (SWCNT) to connect the AuNP

to a macroscopic electrode. Carbodiimide chemistry has previously been applied to the coupling of proteins to SWCNTs. They are just the right size to couple to lysine residues at the edge of the GR active site, once the SWCNTs have been oxidized with nitric acid to form carboxylic acids at their termini. Again, nanoparticle formation would be performed both before and after SWCNT coupling to test for different effects on electrical connection.

[0087] LiMo₃Se₃ nanowires. In a different vein, others have demonstrated that inexpensive, water soluble LiMo₃Se₃ nanowires can be used as electrical connectors between gold nanoparticles and a macroscopic electrode. The LiMo₃Se₃ nanowires bind to gold nanoparticles via covalent Au-Se interactions. Highly conductive films of the LiMo₃Se₃-AuNP composite can be easily fabricated on silicon, gold or glass substrates by drop coating the corresponding solutions. There are at least two approaches for application of the LiMo₃Se₃ nanowires.

[0088] The first approach is to form LiMo₃Se₃-AuNP composite with ~2 nm AuNPs (which have been shown to bind to the GR active site), followed by attachment of GR to the AuNPs in the presence of NADPH. Binding of the GR-AuNP to the nanowires will be determined by electron microscopy and/or atomic force microscopy. This nanocomposite will then be adsorbed to either graphite or gold electrodes to test for electrical connection using standard electrochemical experiments (*eg* cyclic voltammetry).

[0089] The second approach is to adsorb the GR-AuNP conjugate to the LiMo₃Se₃ nanowires in a manner similar to that done previously with simple citrate coated AuNPs. Briefly, low pH and/or high ionic strength facilitates the adhesion process since charge repulsion between the anion-coated surface of the AuNP and the anionic surface of the MoSe nanowires is a barrier to adhesion. The pH of the GR-AuNP solution can be safely lowered to ~5 without loss of enzyme activity and high ionic strength can be achieved in two ways. The first will be to use 1 M TEA-HCl as a buffer. At the low pH, this will largely be in the salt form. A second way is to use ammonium sulfate, which is commonly used in the purification of enzymes. The GR-AuNP solution will be added to the LiMo₃Se₃ nanowire solution and then slowly add ammonium sulfate until the enzyme precipitates as an adduct with the nanowires.

[0090] Direct connection. The following experiments will also be performed in order to connect GR electrically to a gold electrode. Site-directed mutagenesis has already been employed to add a 5×Cys tail to the C-terminus of GR. The C-terminus is optimally located

at the entrance to the GR active site such that chemiadsorption of the 5×Cys tail to a gold surface will orient the active site for good electrical contact between FAD and the surface via an active site AuNP. This is illustrated in Figure 10. The AuNP will be formed *in situ* after attachment to the gold surface to avoid interaction of the 5×Cys tail with the active site bound AuNP.

[0091] A second approach for connecting to a gold surface involves forming a self-assembled monolayer of 1,4-benzenedithiol on the surface followed by addition of GR with an active site bound AuNP. The free sulfhydryls on the monolayer will chemiadsorb to the AuNP in the GR active site, providing a π electron pathway for facile conduction of electrons from FAD to the electrode. A variation on this experiment will be attempted in which the 1,4-benzenedithiol is doped with a small amount of 5-thiopentanoic acid, which will allow covalent attachment of GR via carbodiimide chemistry to strengthen the interaction of the enzyme with the electrode.

[0092] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, one of skill in the art will appreciate that certain changes and modifications may be practiced within the scope of the appended claims. In addition, each reference provided herein is incorporated by reference in its entirety to the same extent as if each reference was individually incorporated by reference.

WHAT IS CLAIMED IS:

1. A method of preparing a nanoparticle, comprising:
 - a) providing at least one redox-active enzyme-coenzyme complex;
 - b) contacting said enzyme-coenzyme complex with a first metal ion and an electron-donor such that said enzyme-coenzyme complex repetitively catalyzes the reduction of said metal ion thereby preparing said nanoparticle bound to said enzyme-coenzyme complex via repetitive metal ion reduction by said enzyme-coenzyme complex; and
 - c) collecting said nanoparticle.
2. The method of claim 1, further comprising the following step:
 - d) repeating step b) with a second metal ion.
3. The method of claim 1, wherein said enzyme-coenzyme complex is a member of the pyridine nucleotide-disulfide oxidoreductase family of enzymes.
4. The method of claim 3, wherein said enzyme-coenzyme complex is a member selected from the group consisting of thioredoxin reductase, lipoamide dehydrogenase, trypanothione reductase, coenzyme A disulfide reductase, alkyl hydroperoxide reductase, NADH oxidase, NADH peroxidase, mercuric ion reductase and glutathione reductase.
5. The method of claim 4, wherein said enzyme-coenzyme complex is glutathione reductase.
6. The method of claim 1, wherein the coenzyme of said enzyme-coenzyme complex is flavin adenine dinucleotide.
7. The method of claim 1, wherein said first metal ion is a member selected from the group consisting of Au (III), Pt (IV), Co (II), Ni (II), Fe (III), Ag (I) and Cd (II).
8. The method of claim 7, where said first metal ion is Au (III).
9. The method of claim 7, where said first metal ion is Pt (IV).

10. The method of claim 1, wherein said electron-donor is nicotinamide adenine dinucleotide (phosphate).
11. The method of claim 1, wherein said collecting step comprises releasing said nanoparticle from said enzyme-coenzyme complex using a sulfhydryl-containing reagent.
12. The method of claim 2, wherein said second metal ion is a member selected from the group consisting of Pt (IV), Co (II), Ni (II) and Fe (III).
13. The method of claim 1, wherein said enzyme-coenzyme complex is attached to a solid support selected from the group consisting of agarose, cyanogen bromide-activated agarose, Sephadex®, cellulose, dextran, starch, chitin, glass, polystyrene, polyacrylamide, polyethyleneglycol, polyethyleneimine, graphite, silica, silica gel and hydroxyapatite (calcium phosphate).
14. An electron transfer system comprising the following elements in electrical connection: an electron donor or acceptor, a redox-active enzyme-coenzyme complex, a nanoparticle, and an electrode, wherein said metal nanoparticle is grown from said enzyme-coenzyme complex via repetitive metal ion reduction by said enzyme-coenzyme complex.
15. The electron transfer system of claim 14, wherein said electron donor or acceptor is nicotinamide adenine dinucleotide phosphate.
16. The electron transfer system of claim 14, wherein said enzyme-coenzyme complex is glutathione reductase.
17. The electron transfer system of claim 14, wherein said nanoparticle comprises at least one metal selected from the group consisting of Au (III), Pt (IV), Co (II), Ni (II), Fe (III), Ag (I) and Cd (II).
18. The electron transfer system of claim 14, wherein said enzyme-coenzyme complex and said nanoparticle are electrically connected to said electrode via a nanotube or nanowire.

19. The electron transfer system of claim 18, wherein said nanotube is a single-walled carbon nanotube.

20. The electron transfer system of claim 18, wherein said nanowire is a LiMo_3Se_3 nanowire.

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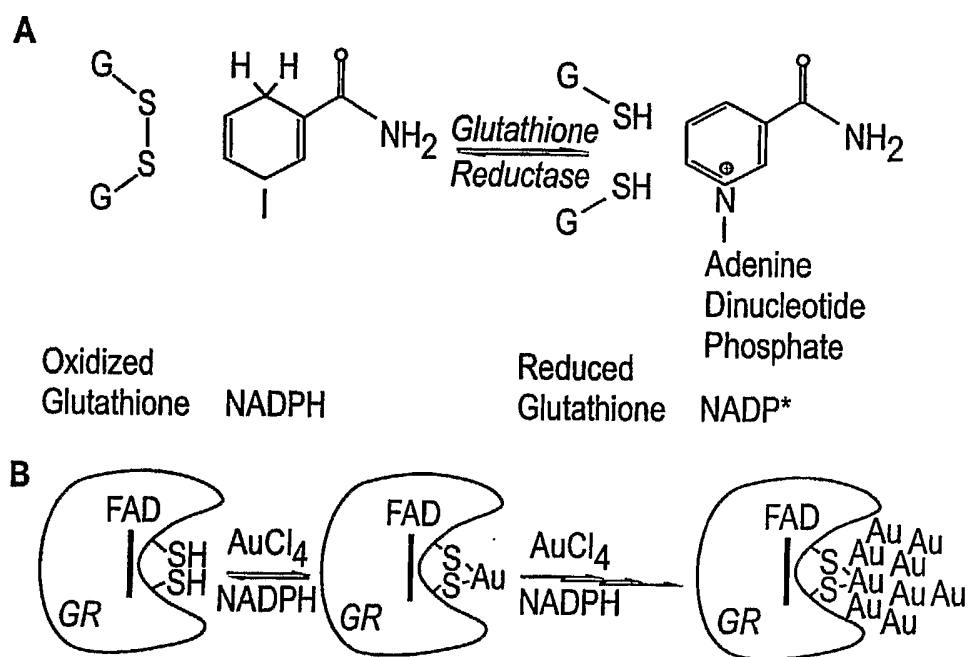


FIG. 1

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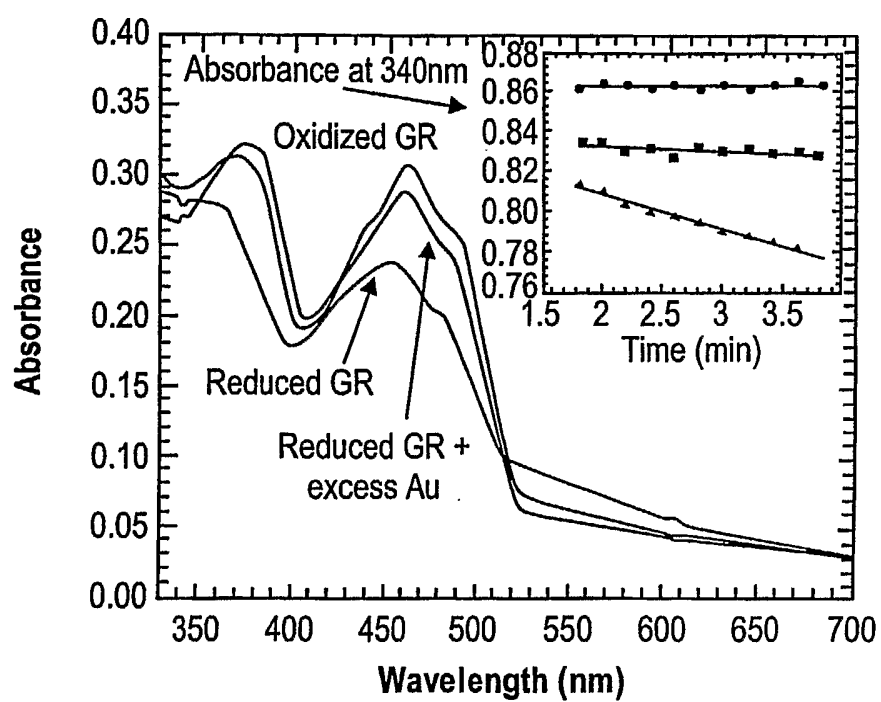


FIG. 2

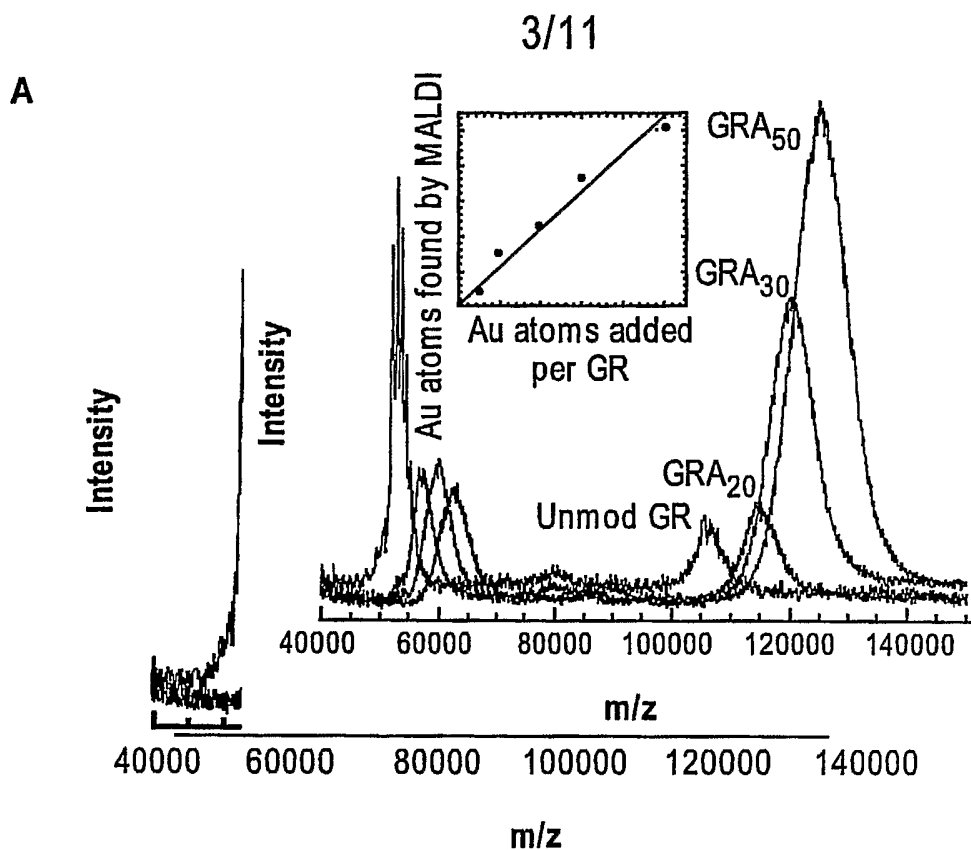


FIG. 3

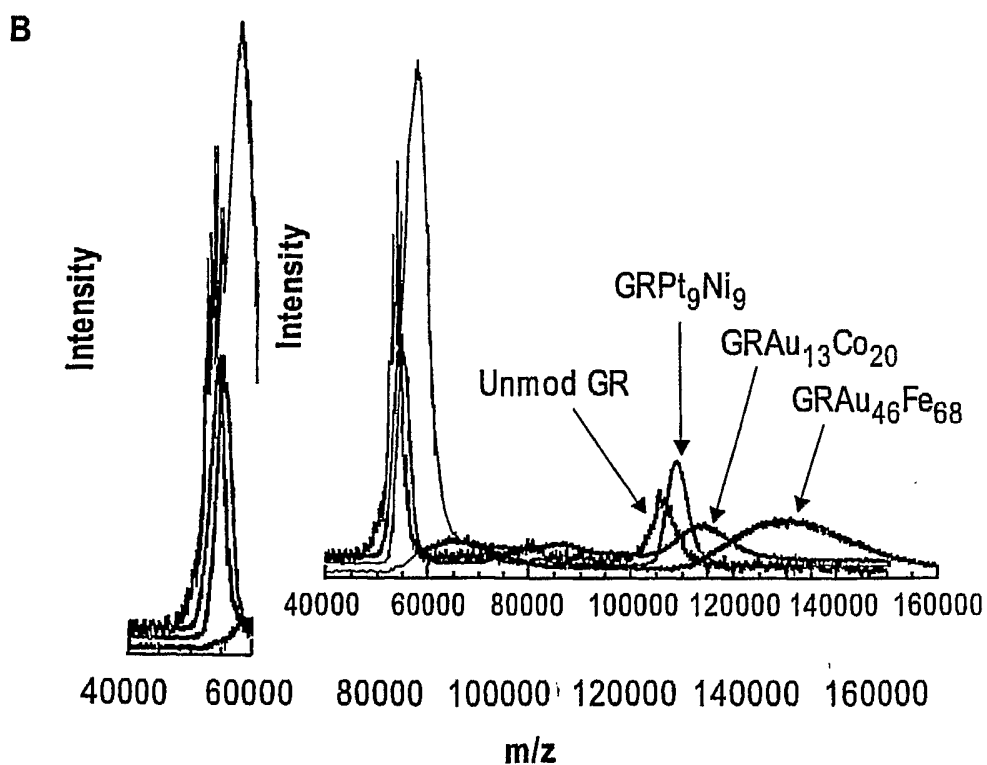


FIG. 4

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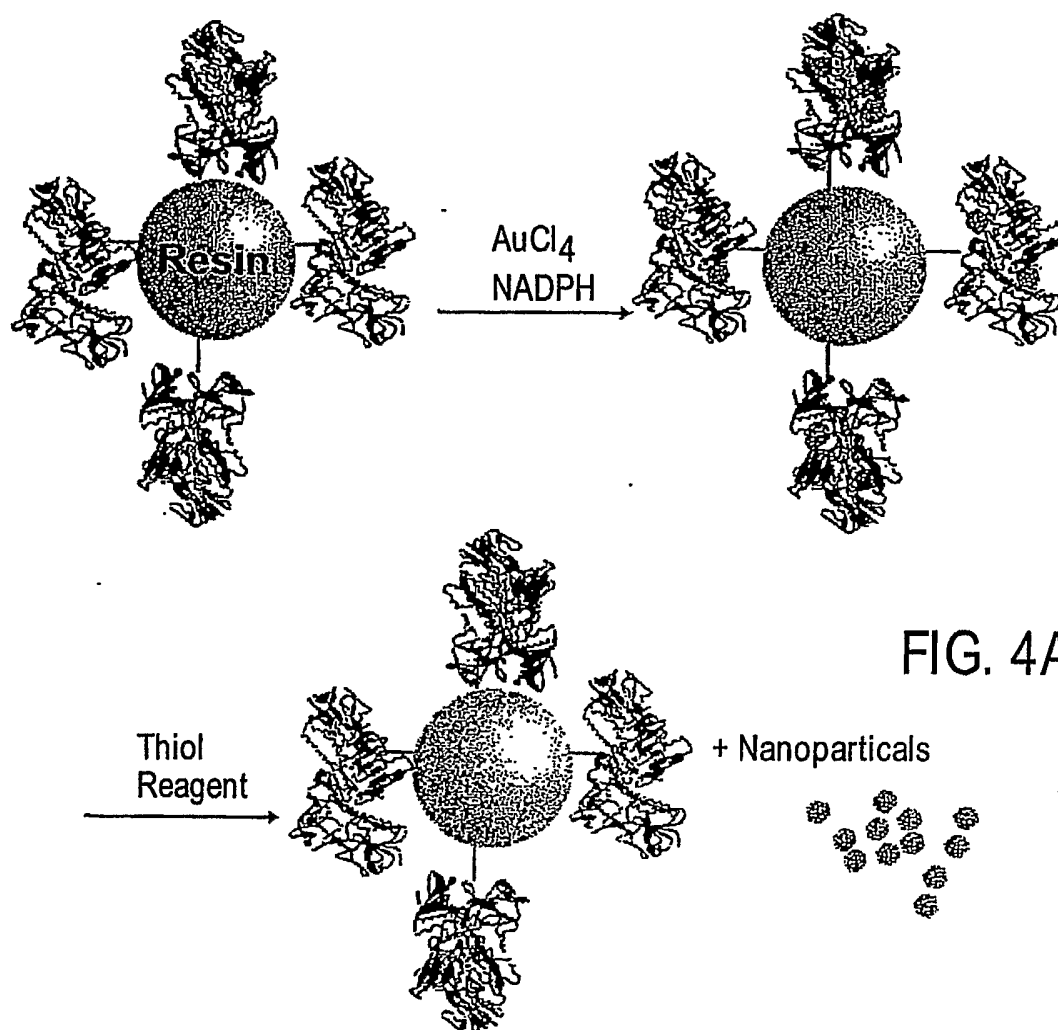
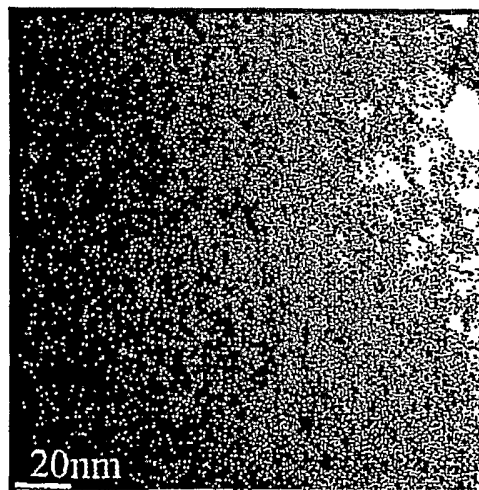


FIG. 4A

FIG. 4B



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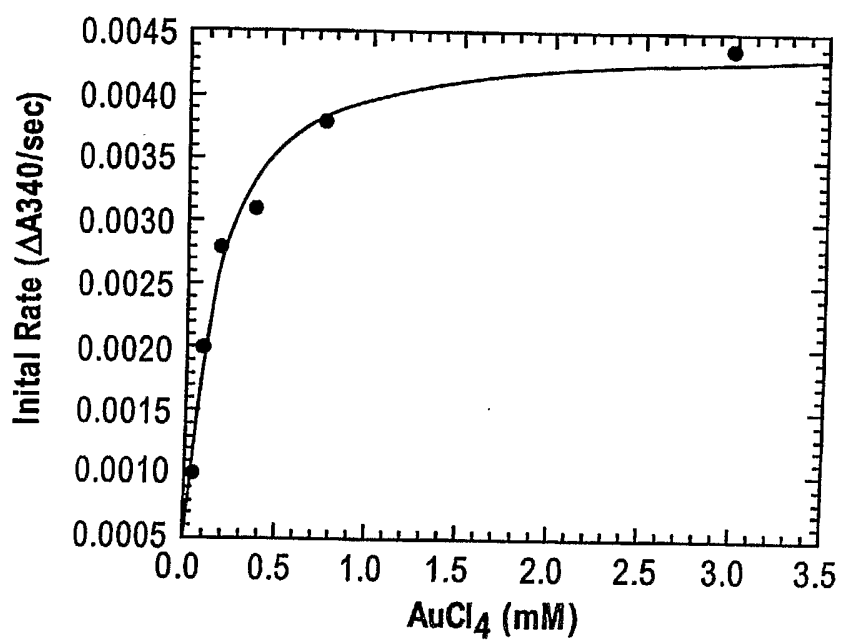


FIG. 5

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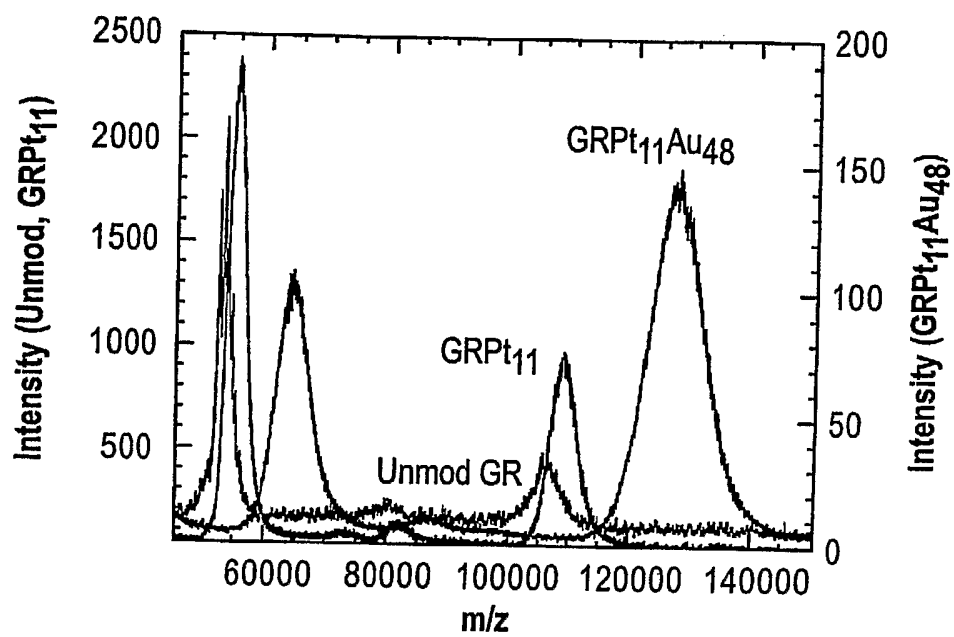


FIG. 6

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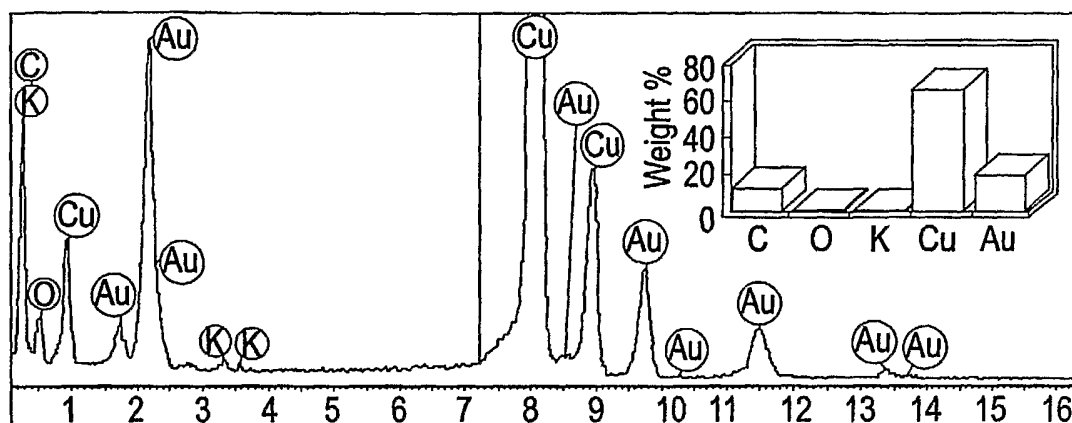


FIG. 7

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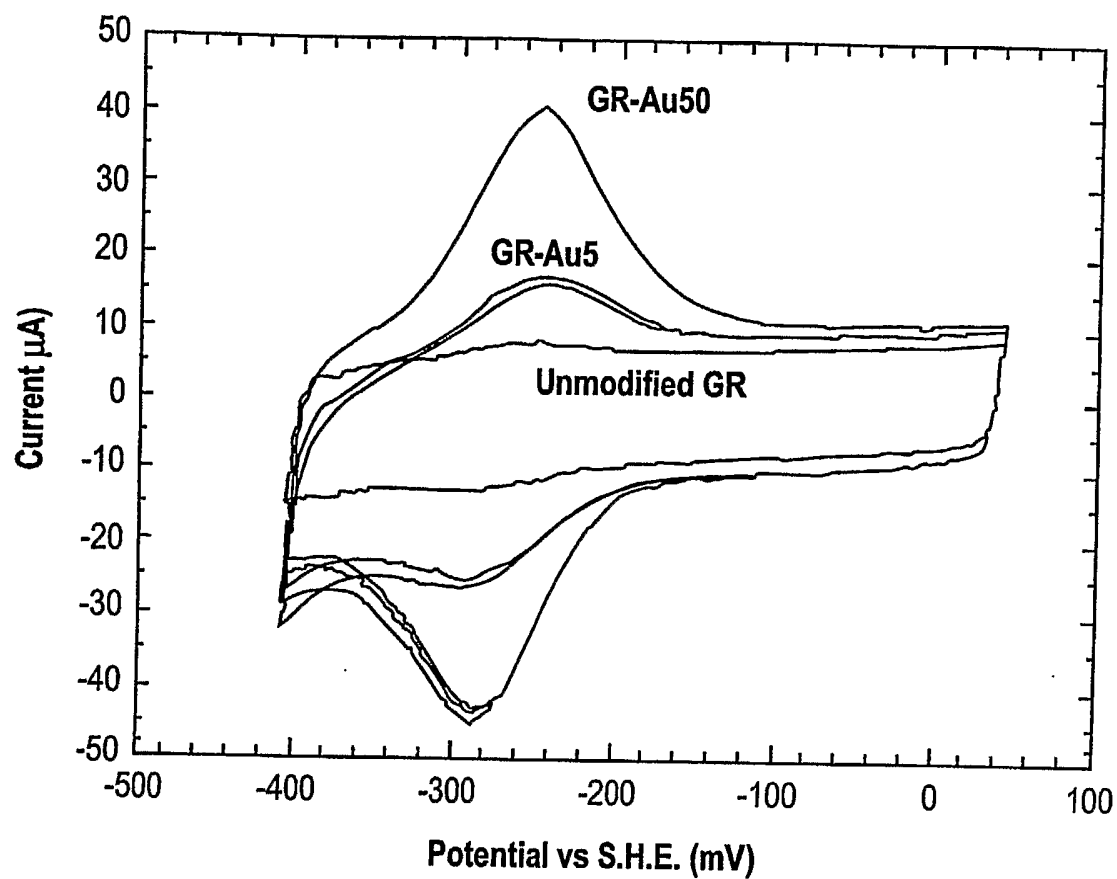


FIG. 8

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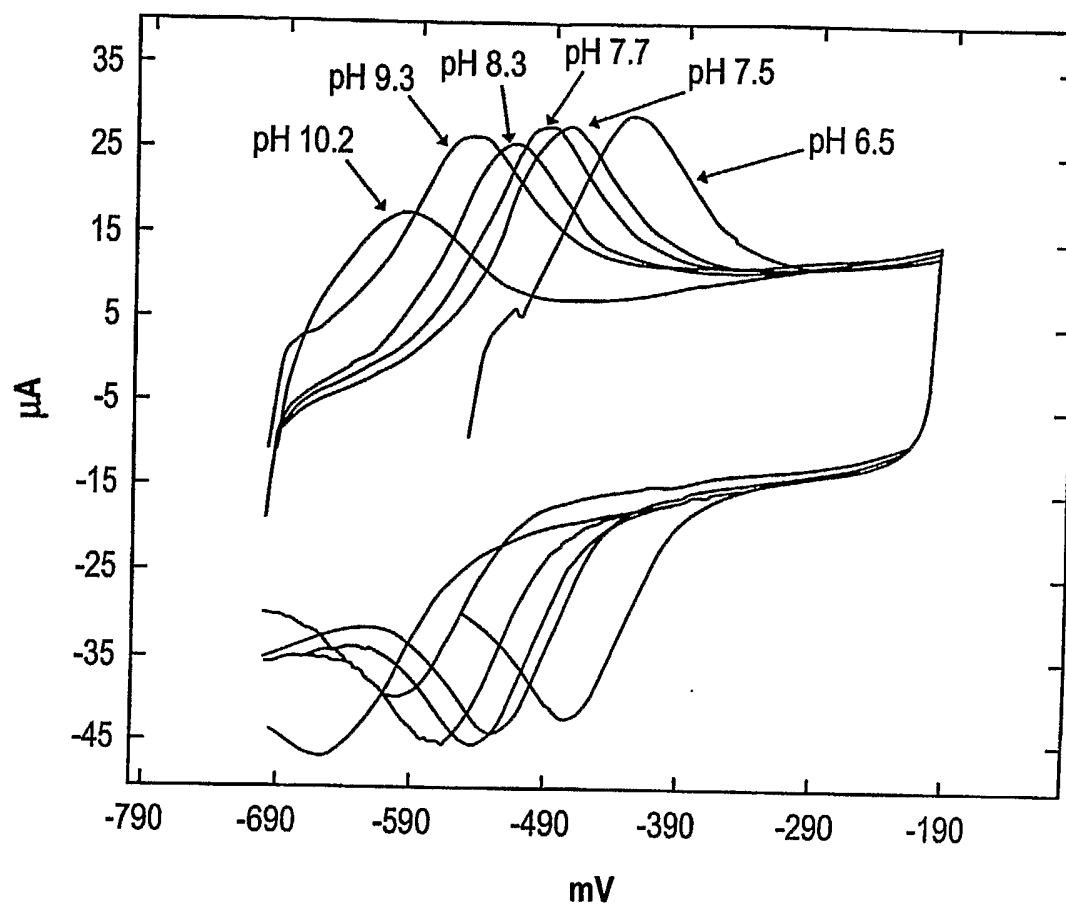


FIG. 9

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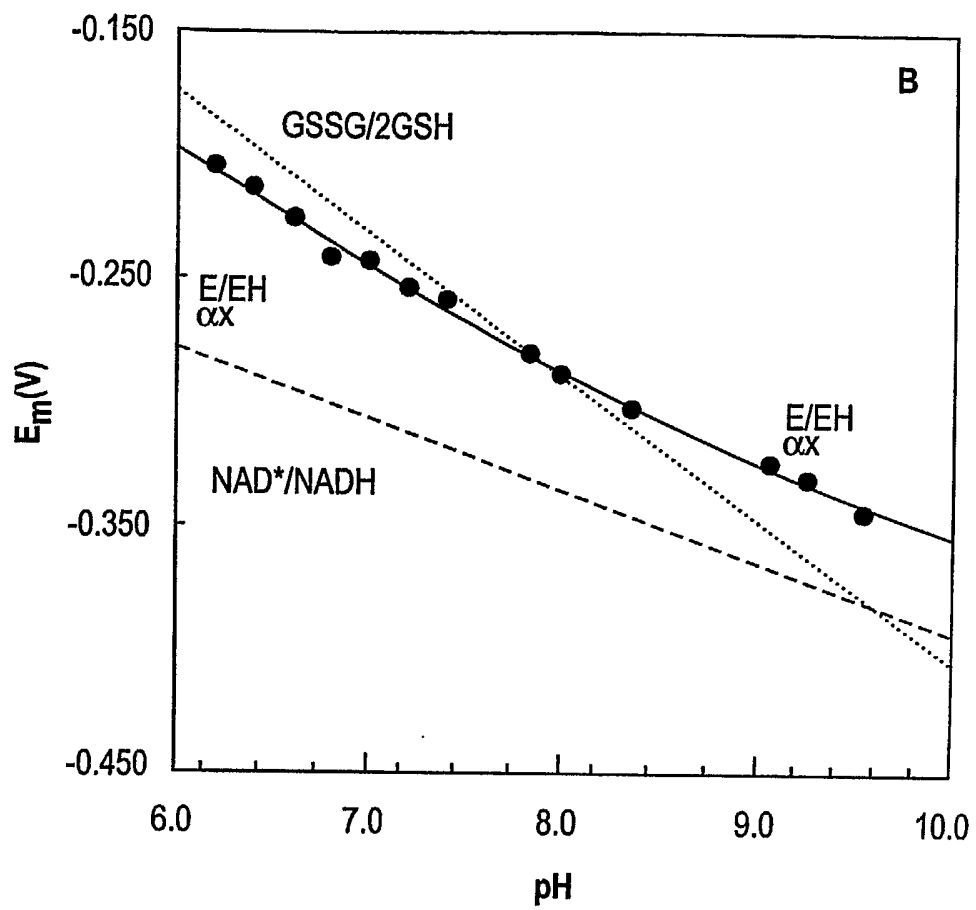


FIG. 10

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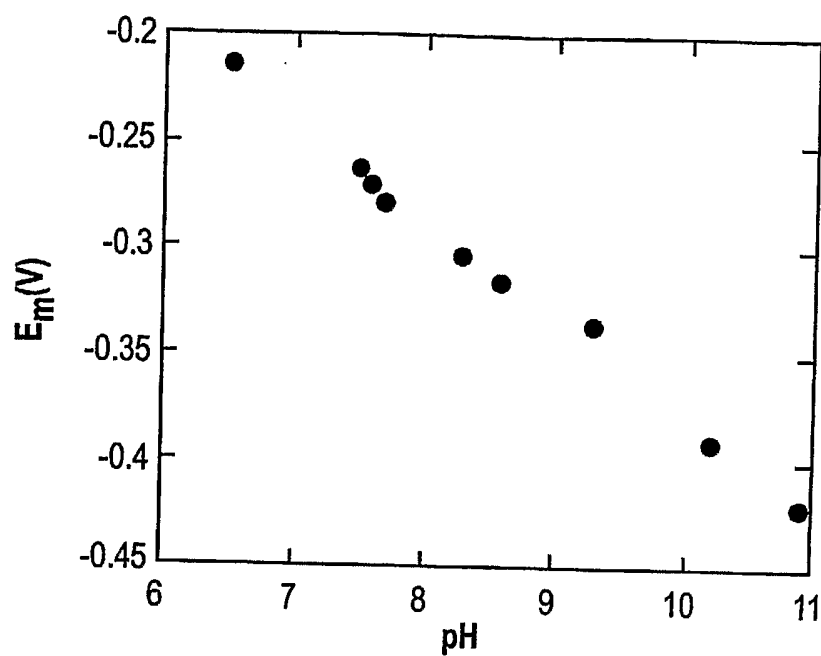


FIG. 11