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(54) Title: COPPER DEHYDROGENASES ENGINEERED FROM MULTICOPPER OXIDASES AND THEIR BIOELECTROCHEMICAL APPLICATIONS

(57) Abstract: Described are copper dehydrogenase enzymes that are engineered from multicopper oxidases to have reduced oxidase activity. The oxygen-insensitive copper dehydrogenases catalyze the dehydrogenation of a phenolic substrate on an electrode to generate electrical current. Compositions, devices, kits, and methods are disclosed for assaying L-DOPA with a copper dehydrogenase. Anodes, enzyme fuel cells and batteries are disclosed with the copper dehydrogenase immobilized with a substrate.



COPPER DEHYDROGENASES ENGINEERED FROM MULTICOPPER OXIDASES AND THEIR BIOELECTROCHEMICAL APPLICATIONS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority to U.S. Provisional Application No. 63/505,267, filed May 31, 2023, which is hereby incorporated by reference in its entirety.

REFERENCE TO A SEQUENCE LISTING

[0002] The Sequence Listing written in file name 614164SEQLIST.xml is 46.9 kilobytes, was created on May 29, 2024, and is hereby incorporated by reference.

BACKGROUND

[0003] Multicopper oxidases (MCOs) belong to a protein superfamily of enzymes which catalyze the four-electron reduction of oxygen to water by the oxidation of various substrates. They catalyze the one-electron oxidation of a broad range of compounds including substituted phenols, arylamines and aromatic thiols. MCOs contain at least four copper atoms, which are classified as type 1 (T1), type 2 (T2) or type 3 (T3). The oxidation of a substrate starts at a T1 mononuclear copper center, then electrons are transferred internally to the trinuclear copper center T2/T3 where oxygen (O₂) is reduced by four electrons, yielding two water molecules. MCOs consist of four enzyme families: laccases (EC 1.10.3.2), ascorbate oxidases (EC 1.10.3.3), ferroxidases (EC 1.16.3.1) and ceruloplasmin (EC 1.16.3.1). Due to their broad substrate scope and stability, there is interest in the use of MCOs, especially fungal and bacterial laccases, for biorefinery and bioelectrochemical applications.

[0004] Tyrosinase and horseradish peroxidase have been explored for the development of enzyme sensors to monitor phenolic compounds. However, tyrosinase suffers from low stability and is significantly inhibited by reaction products, while horseradish peroxidase requires the presence of hydrogen peroxide for catalytic activity. These disadvantages have limited the practical application of tyrosinase and horseradish peroxidase sensors. Native (e.g., wild-type) MCOs, including laccases, use oxygen as an electron acceptor. However, for certain biosensing applications, such as monitoring the presence of substituted phenols, arylamines and aromatic thiols, the reaction with oxygen is always competing with the electron transfer to synthetic electron

acceptors. This activity towards oxygen interferes with direct electron transfer (DET) to the electrode and thus the reductive half reaction of the anode which monitors the oxidation of the phenolic substrate. There remains a need for the development of enzymes with improved capabilities for DET to an electrode for applications as enzyme sensors and fuel cells.

[0005] Monitoring a phenolic compound during the treatment of patients with Parkinson's disease (PD) would be one useful biomedical application of an effective enzyme sensor. PD is a chronic illness that affects more than 10 million people worldwide and clinically presents as the difficulty to speak, swallow, tremors, slowness, mood disorders, and schizophrenia. PD biologically presents through the degeneration of dopaminergic neurons in the substantia nigra of the brain which leads to low production of dopamine. The most effective currently used treatment for PD is the phenolic compound levodopa (l-3,4-dihydroxyphenylalanine; L-DOPA), a dopamine precursor capable of crossing the blood-brain barrier, which is later converted into dopamine. However, there is no standardized L-DOPA measurement platform, so most L-DOPA dosing must rely on patient feedback and other subjective measures. This dosing system presents many challenges further complicated by the fact that L-DOPA has a short therapeutic window and is cytotoxic in high concentrations.

[0006] Accurate detection of L-DOPA would allow for improved dosing and enable better management of middle and late-stage Parkinson's symptoms, which would dramatically improve quality of life for millions worldwide. There is great need for a selective and continuous L-DOPA sensing system to better treat patients with PD. As described herein, copper dehydrogenase enzymes engineered from multicopper oxidases were developed to address these needs.

BRIEF SUMMARY

[0007] Compositions, enzymes, devices, kits, and methods are provided for assaying phenolic compounds in a sample from a subject.

[0008] Compositions, enzymes, devices, kits, and methods are provided for assaying L-DOPA in a sample from a subject.

[0009] One embodiment is a copper dehydrogenase engineered from a multicopper oxidase to have with reduced oxidase activity as compared to a wild-type multicopper oxidase.

[0010] In embodiments, the oxidase activity of the copper dehydrogenase is reduced by at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, at least 92%, at least

93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% as compared to the oxidase activity of the wild-type multicopper oxidase.

[0011] In embodiments, the reduced oxidase activity results from modification at one or more amino acid residues comprising type 2 (T2) and/or type 3 (T3) copper atom ligands.

[0012] In some embodiments, the T2 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 64 and 366 of SEQ ID NO: 7, and the T3 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 66, 104, 106, 368, 431, and 433 of SEQ ID NO: 7.

[0013] In some embodiments, the T2 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 101 and 446 of SEQ ID NO: 19, and the T3 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 103, 141, 143, 448, 498 and 500 of SEQ ID NO: 19.

[0014] In some embodiments, the T2 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 105 and 422 of SEQ ID NO: 20, and the T3 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 107, 153, 155, 424, 491 and 493 of SEQ ID NO: 20.

[0015] In some embodiments, the T2 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 95 and 400 of SEQ ID NO: 21, and the T3 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 97, 135, 137, 402, 449 and 451 of SEQ ID NO: 21.

[0016] In some embodiments, the T2 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 132 and 439 of SEQ ID NO: 22, and the T3 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 134, 171, 173, 441, 494 and 451 of SEQ ID NO: 22.

[0017] In some embodiments, the T2 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 103 and 422 of SEQ ID NO: 23, and the T3 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 105, 151, 153, 424, 491 and 493 of SEQ ID NO: 23.

[0018] In some embodiments, the T2 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 103 and 421 of SEQ ID NO: 24, and the

T3 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 151, 153, 423, 490 and 492 of SEQ ID NO: 24.

[0019] In some embodiments, the T2 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 145 and 511 of SEQ ID NO: 25, and the T3 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 147, 185, 187, 513, 586 and 588 of SEQ ID NO: 25.

[0020] In some embodiments, the T2 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 145 and 514 of SEQ ID NO: 26, and the T3 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 147, 185, 187, 516, 589 and 591 of SEQ ID NO: 26.

[0021] In embodiments, the copper dehydrogenase comprises increased dehydrogenase activity towards a substrate as compared to a wild-type multicopper oxidase.

[0022] In embodiments, the dehydrogenase activity of the copper dehydrogenase is increased by at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 100% as compared to the dehydrogenase activity of the wild-type multicopper oxidase.

[0023] In one embodiment, the substrate is L-DOPA.

[0024] A further embodiment is a copper dehydrogenase comprising a sequence having at least 90% sequence identity to any one of SEQ ID NOs:1-6, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 64, 66, 104, 106, 366, 368, 431, and 433 of SEQ ID NO: 7 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 7.

[0025] A further embodiment is a copper dehydrogenase comprising a sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 99.9% sequence identity to SEQ ID NO:19, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 101, 103, 141, 143, 446, 448, 498 and 500 of SEQ ID NO: 19 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 19.

[0026] A further embodiment is a copper dehydrogenase comprising a sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least

97%, at least 98%, at least 99%, at least 99.5%, or at least 99.9% sequence identity to SEQ ID NO:20, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 105, 107, 153, 155, 422, 424, 491 and 493 of SEQ ID NO: 20 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 20.

[0027] A further embodiment is a copper dehydrogenase comprising a sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 99.9% sequence identity to SEQ ID NO:21, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 95, 97, 135, 137, 400, 402, 449 and 451 of SEQ ID NO: 21 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 21.

[0028] A further embodiment is a copper dehydrogenase comprising a sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 99.9% sequence identity to SEQ ID NO:22, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 132, 134, 171, 173, 439, 441, 494 and 496 of SEQ ID NO: 22 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 22.

[0029] A further embodiment is a copper dehydrogenase comprising a sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 99.9% sequence identity to SEQ ID NO:23, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 103, 105, 151, 153, 422, 424, 491 and 493 of SEQ ID NO: 23 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 23.

[0030] A further embodiment is a copper dehydrogenase comprising a sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 99.9% sequence identity to SEQ ID NO:24, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 103, 105, 151, 153, 421, 423, 490 and

492 of SEQ ID NO: 24 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 24.

[0031] A further embodiment is a copper dehydrogenase comprising a sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 99.9% sequence identity to SEQ ID NO:25, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 145, 147, 185, 187, 511, 513, 586 and 588 of SEQ ID NO: 25 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 25.

[0032] A further embodiment is a copper dehydrogenase comprising a sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 99.9% sequence identity to SEQ ID NO:26, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 145, 147, 185, 187, 514, 516, 589 and 591 of SEQ ID NO: 26 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 26.

[0033] Another embodiment is a copper dehydrogenase comprising a modification of one or more histidine residues selected from: (a) a position corresponding to position 66 of the amino acid sequence set forth in SEQ ID NO:7; (b) a position corresponding to position 104 of the amino acid sequence set forth in SEQ ID NO:7; (c) a position corresponding to position 368 of the amino acid sequence set forth in SEQ ID NO:7; and (d) a position corresponding to position 431 of the amino acid sequence set forth in SEQ ID NO:7.

[0034] Another embodiment is a copper dehydrogenase comprising a modification of one or more histidine residues selected from: (a) a position corresponding to position 103 of the amino acid sequence set forth in SEQ ID NO: 19; (b) a position corresponding to position 141 of the amino acid sequence set forth in SEQ ID NO: 19; (c) a position corresponding to position 448 of the amino acid sequence set forth in SEQ ID NO: 19; and (d) a position corresponding to position 498 of the amino acid sequence set forth in SEQ ID NO: 19.

[0035] Another embodiment is a copper dehydrogenase comprising a modification of one or more histidine residues selected from: (a) a position corresponding to position 107 of the amino acid sequence set forth in SEQ ID NO: 20; (b) a position corresponding to position 153 of the

amino acid sequence set forth in SEQ ID NO: 20; (c) a position corresponding to position 424 of the amino acid sequence set forth in SEQ ID NO: 20; and (d) a position corresponding to position 491 of the amino acid sequence set forth in SEQ ID NO: 20.

[0036] Another embodiment is a copper dehydrogenase comprising a modification of one or more histidine residues selected from: (a) a position corresponding to position 97 of the amino acid sequence set forth in SEQ ID NO: 21; (b) a position corresponding to position 135 of the amino acid sequence set forth in SEQ ID NO: 21; (c) a position corresponding to position 402 of the amino acid sequence set forth in SEQ ID NO: 21; and (d) a position corresponding to position 449 of the amino acid sequence set forth in SEQ ID NO: 21.

[0037] Another embodiment is a copper dehydrogenase comprising a modification of one or more histidine residues selected from: (a) a position corresponding to position 134 of the amino acid sequence set forth in SEQ ID NO: 22; (b) a position corresponding to position 171 of the amino acid sequence set forth in SEQ ID NO: 22; (c) a position corresponding to position 441 of the amino acid sequence set forth in SEQ ID NO: 22; and (d) a position corresponding to position 494 of the amino acid sequence set forth in SEQ ID NO: 22.

[0038] Another embodiment is a copper dehydrogenase comprising a modification of one or more histidine residues selected from: (a) a position corresponding to position 105 of the amino acid sequence set forth in SEQ ID NO: 23; (b) a position corresponding to position 151 of the amino acid sequence set forth in SEQ ID NO: 23; (c) a position corresponding to position 424 of the amino acid sequence set forth in SEQ ID NO: 23; and (d) a position corresponding to position 491 of the amino acid sequence set forth in SEQ ID NO: 23.

[0039] Another embodiment is a copper dehydrogenase comprising a modification of one or more histidine residues selected from: (a) a position corresponding to position 105 of the amino acid sequence set forth in SEQ ID NO: 24; (b) a position corresponding to position 151 of the amino acid sequence set forth in SEQ ID NO: 24; (c) a position corresponding to position 423 of the amino acid sequence set forth in SEQ ID NO: X6; and (d) a position corresponding to position 490 of the amino acid sequence set forth in SEQ ID NO: 24.

[0040] Another embodiment is a copper dehydrogenase comprising a modification of one or more histidine residues selected from: (a) a position corresponding to position 147 of the amino acid sequence set forth in SEQ ID NO: 25; (b) a position corresponding to position 185 of the amino acid sequence set forth in SEQ ID NO: 25; (c) a position corresponding to position 513 of

the amino acid sequence set forth in SEQ ID NO: 25; and (d) a position corresponding to position 586 of the amino acid sequence set forth in SEQ ID NO: 25.

[0041] Another embodiment is a copper dehydrogenase comprising a modification of one or more histidine residues selected from: (a) a position corresponding to position 147 of the amino acid sequence set forth in SEQ ID NO: 26; (b) a position corresponding to position 185 of the amino acid sequence set forth in SEQ ID NO: 26; (c) a position corresponding to position 516 of the amino acid sequence set forth in SEQ ID NO: 26; and (d) a position corresponding to position 589 of the amino acid sequence set forth in SEQ ID NO: 26.

[0042] In embodiments, the modification is a substitution with a different amino acid residue or a deletion.

[0043] In one embodiment, the copper dehydrogenase comprises a modification at one or more amino acid residues at a position selected from: (a) a position corresponding to position 66 of the amino acid sequence set forth in SEQ ID NO:7, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; (b) a position corresponding to position 104 of the amino acid sequence set forth in SEQ ID NO:7, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; (c) a position corresponding to position 368 of the amino acid sequence set forth in SEQ ID NO:7, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; and (d) a position corresponding to position 431 of the amino acid sequence set forth in SEQ ID NO:7, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue.

[0044] In one embodiment, the copper dehydrogenase comprises a modification at one or more amino acid residues at a position selected from: (a) a position corresponding to position 103 of the amino acid sequence set forth in SEQ ID NO: 19, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; (b) a position corresponding to position 141 of the amino acid sequence set forth in SEQ ID NO: 19, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; (c) a position corresponding to position 448 of the amino acid sequence set forth in SEQ ID NO: 19, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; and (d) a position corresponding to position 498 of the amino acid

sequence set forth in SEQ ID NO: 19, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue.

[0045] In one embodiment, the copper dehydrogenase comprises a modification at one or more amino acid residues at a position selected from: (a) a position corresponding to position 107 of the amino acid sequence set forth in SEQ ID NO: 20, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; (b) a position corresponding to position 153 of the amino acid sequence set forth in SEQ ID NO: 20, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; (c) a position corresponding to position 424 of the amino acid sequence set forth in SEQ ID NO: 20, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; and (d) a position corresponding to position 491 of the amino acid sequence set forth in SEQ ID NO: 20, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue.

[0046] In one embodiment, the copper dehydrogenase comprises a modification at one or more amino acid residues at a position selected from: (a) a position corresponding to position 97 of the amino acid sequence set forth in SEQ ID NO: 21, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; (b) a position corresponding to position 135 of the amino acid sequence set forth in SEQ ID NO: 21, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; (c) a position corresponding to position 402 of the amino acid sequence set forth in SEQ ID NO: 21, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; and (d) a position corresponding to position 449 of the amino acid sequence set forth in SEQ ID NO: 21, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue.

[0047] In one embodiment, the copper dehydrogenase comprises a modification at one or more amino acid residues at a position selected from: (a) a position corresponding to position 134 of the amino acid sequence set forth in SEQ ID NO: 22, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; (b) a position corresponding to position 171 of the amino acid sequence set forth in SEQ ID NO: 22, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; (c) a position corresponding to position 441 of the amino acid sequence set forth in SEQ

ID NO: 22, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; and (d) a position corresponding to position 494 of the amino acid sequence set forth in SEQ ID NO: 22, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue.

[0048] In one embodiment, the copper dehydrogenase comprises a modification at one or more amino acid residues at a position selected from: (a) a position corresponding to position 105 of the amino acid sequence set forth in SEQ ID NO: 23, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; (b) a position corresponding to position 151 of the amino acid sequence set forth in SEQ ID NO: 23, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; (c) a position corresponding to position 424 of the amino acid sequence set forth in SEQ ID NO: 23, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; and (d) a position corresponding to position 491 of the amino acid sequence set forth in SEQ ID NO: 23, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue.

[0049] In one embodiment, the copper dehydrogenase comprises a modification at one or more amino acid residues at a position selected from: (a) a position corresponding to position 105 of the amino acid sequence set forth in SEQ ID NO: 24, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; (b) a position corresponding to position 151 of the amino acid sequence set forth in SEQ ID NO: 24, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; (c) a position corresponding to position 423 of the amino acid sequence set forth in SEQ ID NO: 24, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; and (d) a position corresponding to position 490 of the amino acid sequence set forth in SEQ ID NO: 24, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue.

[0050] In one embodiment, the copper dehydrogenase comprises a modification at one or more amino acid residues at a position selected from: (a) a position corresponding to position 147 of the amino acid sequence set forth in SEQ ID NO: 25, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; (b) a position corresponding to position 185 of the amino acid sequence set forth in SEQ ID NO: 25, wherein

the modification includes a substitution of the wild-type amino acid residue with an alanine residue; (c) a position corresponding to position 513 of the amino acid sequence set forth in SEQ ID NO: 25, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; and (d) a position corresponding to position 586 of the amino acid sequence set forth in SEQ ID NO: 25, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue.

[0051] In one embodiment, the copper dehydrogenase comprises a modification at one or more amino acid residues at a position selected from: (a) a position corresponding to position 147 of the amino acid sequence set forth in SEQ ID NO: 26, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; (b) a position corresponding to position 185 of the amino acid sequence set forth in SEQ ID NO: 26, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; (c) a position corresponding to position 516 of the amino acid sequence set forth in SEQ ID NO: 26, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; and (d) a position corresponding to position 589 of the amino acid sequence set forth in SEQ ID NO: 26, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue.

[0052] A further embodiment is a copper dehydrogenase comprising a modification at one or more amino acid residues at a position selected from: (a) a position corresponding to position 368 of the amino acid sequence set forth in SEQ ID NO:7, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; and (b) a position corresponding to position 431 of the amino acid sequence set forth in SEQ ID NO:7, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue.

[0053] A further embodiment is a copper dehydrogenase comprising a modification at one or more amino acid residues at a position selected from: (a) a position corresponding to position 448 of the amino acid sequence set forth in SEQ ID NO: 19, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; and (b) a position corresponding to position 498 of the amino acid sequence set forth in SEQ ID NO: 19, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue.

[0054] A further embodiment is a copper dehydrogenase comprising a modification at one or more amino acid residues at a position selected from: (a) a position corresponding to position 424 of the amino acid sequence set forth in SEQ ID NO: 20, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; and (b) a position corresponding to position 491 of the amino acid sequence set forth in SEQ ID NO: 20, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue.

[0055] A further embodiment is a copper dehydrogenase comprising a modification at one or more amino acid residues at a position selected from: (a) a position corresponding to position 402 of the amino acid sequence set forth in SEQ ID NO: 21, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; and (b) a position corresponding to position 449 of the amino acid sequence set forth in SEQ ID NO: 21, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue.

[0056] A further embodiment is a copper dehydrogenase comprising a modification at one or more amino acid residues at a position selected from: (a) a position corresponding to position 441 of the amino acid sequence set forth in SEQ ID NO: 22, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; and (b) a position corresponding to position 494 of the amino acid sequence set forth in SEQ ID NO: 22, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue.

[0057] A further embodiment is a copper dehydrogenase comprising a modification at one or more amino acid residues at a position selected from: (a) a position corresponding to position 424 of the amino acid sequence set forth in SEQ ID NO: 23, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; and (b) a position corresponding to position 491 of the amino acid sequence set forth in SEQ ID NO: 23, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue.

[0058] A further embodiment is a copper dehydrogenase comprising a modification at one or more amino acid residues at a position selected from: (a) a position corresponding to position 423 of the amino acid sequence set forth in SEQ ID NO: 24, wherein the modification includes a

substitution of the wild-type amino acid residue with an alanine residue; and (b) a position corresponding to position 490 of the amino acid sequence set forth in SEQ ID NO: 24, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue.

[0059] A further embodiment is a copper dehydrogenase comprising a modification at one or more amino acid residues at a position selected from: (a) a position corresponding to position 513 of the amino acid sequence set forth in SEQ ID NO: 25, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; and (b) a position corresponding to position 586 of the amino acid sequence set forth in SEQ ID NO: 25, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue.

[0060] A further embodiment is a copper dehydrogenase comprising a modification at one or more amino acid residues at a position selected from: (a) a position corresponding to position 516 of the amino acid sequence set forth in SEQ ID NO: 26, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; and (b) a position corresponding to position 589 of the amino acid sequence set forth in SEQ ID NO: 26, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue.

[0061] In some embodiments, the copper dehydrogenase comprises a substitution of the wild-type amino acid residue corresponding to position 262 of the amino acid sequence set forth in SEQ ID NO: 7. In some embodiments the wild-type amino acid residue corresponding to position 262 of the amino acid sequence set forth in SEQ ID NO: 7 is a phenylalanine residue, and the substitution comprises changing the wild-type phenylalanine residue to an isoleucine residue.

[0062] Another embodiment is a copper dehydrogenase having at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 99.9% sequence identity to any one of SEQ ID NOs: 1-6.

[0063] Further embodiments are copper dehydrogenases comprising any one of the amino acid sequences set forth in SEQ ID NOs: 1-6.

[0064] A further embodiment is a method of assaying L-DOPA in a sample, the method comprising the steps of: contacting the sample with said copper dehydrogenase; and measuring an amount of L-DOPA.

[0065] In some embodiments, the measurement is an open-circuit potential, chronoamperometry, square-wave voltammetry, or extended gate field effect transistor (EGFET) measurement.

[0066] In some embodiments, the measurement is a continuous measurement.

[0067] One embodiment is a device for assaying L-DOPA in a sample, the device comprising said copper dehydrogenase.

[0068] In embodiments said device further comprises: (I) a three-electrode configuration comprising a working electrode, a reference electrode, and a counter electrode; or (II) a two-electrode configuration comprising a working electrode and a shared reference and a counter electrode.

[0069] In some embodiments one or more electrodes are disk electrodes or needle electrodes.

[0070] In some embodiments one or more electrodes are gold electrodes.

[0071] In some embodiments, said device comprises a sensor strip. In one embodiment, the sensor strip is screen-printed. In some embodiments, the sensor strip is single-use or disposable.

[0072] Another embodiment is a kit for assaying L-DOPA in a sample, the kit comprising said copper dehydrogenase.

[0073] A further embodiment is an enzyme electrode comprising said copper dehydrogenase immobilized on an electrode.

[0074] Another embodiment is an enzyme sensor for assaying L-DOPA comprising said enzyme electrode.

[0075] One embodiment is a polynucleotide encoding a copper dehydrogenase, comprising a modification of one or more amino acid residues selected from: i) a position corresponding to position 66 of the amino acid sequence set forth in SEQ ID NO:7, ii) a position corresponding to position 104 of the amino acid sequence set forth in SEQ ID NO:7, iii) a position corresponding to position 368 of the amino acid sequence set forth in SEQ ID NO:7, and iv) a position corresponding to position 431 of the amino acid sequence set forth in SEQ ID NO:7.

[0076] One embodiment is a polynucleotide encoding a copper dehydrogenase, comprising a modification of one or more amino acid residues selected from: i) a position corresponding to position 103 of the amino acid sequence set forth in SEQ ID NO: 19, ii) a position corresponding to position 141 of the amino acid sequence set forth in SEQ ID NO: 19, iii) a position

corresponding to position 448 of the amino acid sequence set forth in SEQ ID NO: 19, and iv) a position corresponding to position 498 of the amino acid sequence set forth in SEQ ID NO: 19.

[0077] One embodiment is a polynucleotide encoding a copper dehydrogenase, comprising a modification of one or more amino acid residues selected from: i) a position corresponding to position 107 of the amino acid sequence set forth in SEQ ID NO: 20, ii) a position corresponding to position 153 of the amino acid sequence set forth in SEQ ID NO: 20, iii) a position corresponding to position 424 of the amino acid sequence set forth in SEQ ID NO: 20, and iv) a position corresponding to position 491 of the amino acid sequence set forth in SEQ ID NO: 20.

[0078] One embodiment is a polynucleotide encoding a copper dehydrogenase, comprising a modification of one or more amino acid residues selected from: i) a position corresponding to position 97 of the amino acid sequence set forth in SEQ ID NO: 21, ii) a position corresponding to position 135 of the amino acid sequence set forth in SEQ ID NO: 21, iii) a position corresponding to position 402 of the amino acid sequence set forth in SEQ ID NO: 21, and iv) a position corresponding to position 449 of the amino acid sequence set forth in SEQ ID NO: 20.

[0079] One embodiment is a polynucleotide encoding a copper dehydrogenase, comprising a modification of one or more amino acid residues selected from: i) a position corresponding to position 134 of the amino acid sequence set forth in SEQ ID NO: 22, ii) a position corresponding to position 171 of the amino acid sequence set forth in SEQ ID NO: 22, iii) a position corresponding to position 441 of the amino acid sequence set forth in SEQ ID NO: 22, and iv) a position corresponding to position 494 of the amino acid sequence set forth in SEQ ID NO: 22.

[0080] One embodiment is a polynucleotide encoding a copper dehydrogenase, comprising a modification of one or more amino acid residues selected from: i) a position corresponding to position 105 of the amino acid sequence set forth in SEQ ID NO: 23, ii) a position corresponding to position 151 of the amino acid sequence set forth in SEQ ID NO: 23, iii) a position corresponding to position 424 of the amino acid sequence set forth in SEQ ID NO: 23, and iv) a position corresponding to position 491 of the amino acid sequence set forth in SEQ ID NO: 23.

[0081] One embodiment is a polynucleotide encoding a copper dehydrogenase, comprising a modification of one or more amino acid residues selected from: i) a position corresponding to position 105 of the amino acid sequence set forth in SEQ ID NO: 24, ii) a position corresponding to position 151 of the amino acid sequence set forth in SEQ ID NO: 24, iii) a position

corresponding to position 423 of the amino acid sequence set forth in SEQ ID NO: 24, and iv) a position corresponding to position 490 of the amino acid sequence set forth in SEQ ID NO: 24.

[0082] One embodiment is a polynucleotide encoding a copper dehydrogenase, comprising a modification of one or more amino acid residues selected from: i) a position corresponding to position 147 of the amino acid sequence set forth in SEQ ID NO: 25, ii) a position corresponding to position 185 of the amino acid sequence set forth in SEQ ID NO: 25, iii) a position corresponding to position 513 of the amino acid sequence set forth in SEQ ID NO: 25, and iv) a position corresponding to position 586 of the amino acid sequence set forth in SEQ ID NO: 25.

[0083] One embodiment is a polynucleotide encoding a copper dehydrogenase, comprising a modification of one or more amino acid residues selected from: i) a position corresponding to position 147 of the amino acid sequence set forth in SEQ ID NO: 26, ii) a position corresponding to position 185 of the amino acid sequence set forth in SEQ ID NO: 26, iii) a position corresponding to position 516 of the amino acid sequence set forth in SEQ ID NO: 26, and iv) a position corresponding to position 589 of the amino acid sequence set forth in SEQ ID NO: 26.

[0084] Further embodiments are polynucleotides encoding said copper dehydrogenases.

[0085] In some embodiments, said polynucleotide comprises a sequence set forth in any one of SEQ ID NOs: 11-16.

[0086] One embodiment is a method of producing a copper dehydrogenase comprising the steps of: i) culturing a host cell transfected with a vector comprising a nucleic acid sequence encoding the copper dehydrogenase under conditions suitable for expression of the polypeptide from the host cell, and ii) recovering the copper dehydrogenase.

[0087] Another embodiment is an anode comprising an electroconductive material, wherein said copper dehydrogenase is immobilized on the anode and a substrate immobilized on the anode, wherein the substrate can be oxidized by the enzyme.

[0088] In embodiments is said anode, wherein the substrate is formed by depositing a cross-linking reagent. In one embodiment, the cross-linking reagent is glutaraldehyde. In embodiments, the substrate is a phenolic compound. In one embodiment, the phenolic compound is derived from lignin.

[0089] In embodiments, the electroconductive material of the anode is selected from the group consisting of carbon paper, glassy carbon, carbon nanotubes, gold, and palladium.

[0090] A further embodiment is a fuel cell comprising said anode and a cathode, wherein the cathode comprises an electroconductive material and an electron acceptor, wherein the anode and the cathode are in a solution, wherein the cathode is contact with dissolved oxygen.

[0091] In some embodiments, the electron acceptor of the cathode comprises the reduced status of the electron acceptor molecules which can transfer electron to oxygen spontaneously. In some embodiments, the electron acceptor of the cathode comprises a phenazine compound. In embodiments, the phenazine compound comprises phenazine ethosulfate (PES) or phenazine methosulfate (PMS). In some embodiments, the phenazine compound is amine-reactive. In one embodiment, the amine-reactive phenazine compound is N-hydroxysuccinimidylester 1-propoxy-5-ethylphenazinium ethyl sulfate (arPES).

[0092] In some embodiments, the electron acceptor of the cathode comprises a metal complex. In one embodiment, the metal complex is an osmium complex or a ruthenium complex.

[0093] In some embodiments, the electron acceptor of the cathode is immobilized on a protein. In embodiments, the protein is selected from the group consisting of bovine serum albumin (BSA), glucose dehydrogenase (GDH), lactate dehydrogenase (LDH), glucose oxidase (GOx), and lactate oxidase (LOx).

[0094] In some embodiments, the electron acceptor of the cathode is an oxidase enzyme. In some embodiments, the oxidase enzyme of the cathode is a multicopper oxidase. In some embodiments, the multicopper oxidase of the cathode is selected from the group laccase, bilirubin oxidase, ascorbic acid oxidase, and multicopper oxidase derived from *Pyrobaculum aerophilum*. In one embodiment, the multicopper oxidase of the cathode is laccase derived from *Trametes versicolor* or *Aspergillus sp.* In one embodiment, the multicopper oxidase of the cathode is bilirubin oxidase derived from *Myrothecium verrucaria*.

[0095] One embodiment is said fuel cell, wherein the solution of the anode and the solution of the cathode are in the same environment.

[0096] Another embodiment is said fuel cell, wherein the solution of the anode and the solution of the cathode are in separate environments and connected by a salt bridge.

[0097] A further embodiment is a battery comprising said fuel cell and a container. In some embodiments, said battery comprises more than one fuel cell. In one embodiment, the fuel cells are in a series configuration. In another embodiment, the fuel cells are in a parallel configuration. In some embodiments, the fuel cells are stacked.

[0098] In some embodiments, said battery comprises a biodegradable material.

BRIEF DESCRIPTION OF THE FIGURES

[0099] **Figure 1** shows a schematic overview of a multicopper oxidase.

[0100] **Figure 2** shows a summary overview of the engineered copper dehydrogenase and its uses for levodopa (L-DOPA) sensing in several formfactors and biosensing schemes.

[0101] **Figure 3** shows SDS-PAGE analyses of purified *Pyrobaculum aerophilum* derived multicopper oxidase (PaMCO) mutants.

[0102] **Figure 4** shows relative oxidase activity measurements of PaMCO mutants.

[0103] **Figure 5** shows calibration curves of an immobilized PaMCO compared to a PaMCO His396Ala/His459Ala mutant which demonstrate oxygen sensitivity and illustrates the effects of enzyme engineering to improve enzyme dehydrogenase activity for L-DOPA measurement.

[0104] **Figure 6A** shows amperometric raw data and **Figure 6B** shows an amperometric calibration curve of a L-DOPA biosensor employing immobilized PaMCO His396Ala/His459Ala.

[0105] **Figure 7A** shows open circuit potential raw data and **Figure 7B** shows an open circuit potential calibration curve of a L-DOPA biosensor employing immobilized PaMCO His396Ala/His459Ala.

[0106] **Figure 8A** shows square wave voltammetry raw data and **Figure 8B** shows a square wave voltammetry calibration curve of a L-DOPA biosensor employing immobilized PaMCO His396Ala/His459Ala.

[0107] **Figure 9A** shows extended gate field effect transistor (EGFET) raw data and **Figure 9B** shows an EGFET calibration curve of a L-DOPA biosensor employing immobilized PaMCO His396Ala/His459Ala.

[0108] **Figure 10** shows open circuit potential (OCP) bias from the addition of C-DOPA across various L-DOPA concentration profiles.

[0109] **Figure 11** shows chronoamperometry (CA) signal bias from the addition of C-DOPA across various L-DOPA concentration profiles.

[0110] **Figure 12** shows chronoamperometry signal bias from the addition of various interferents at high end physiological concentrations to a 10 μ M L-DOPA solution.

[0111] **Figure 13** shows open circuit potential signal bias from the addition of various interferents at high end physiological concentrations to a 10 μ M L-DOPA solution.

[0112] **Figure 14** shows signal bias from the addition of different interferents within physiological ranges in a L-DOPA sensor that utilizes tyrosinase as its bio-recognition element. All measurements were taken with specific concentrations of the interferents which were added to a solution containing 180 μ M L-DOPA.

[0113] **Figure 15** shows signal bias from the addition of different interferents within physiological ranges, in a L-DOPA sensor that utilizes the engineered PaMCO His396Ala/His459Ala enzyme as its bio-recognition element. All measurements were taken with specific concentrations of the interferents which were added to a solution containing 180 μ M L-DOPA.

[0114] **Figures 16A-16E** show calibration curves of L-DOPA biosensors fabricated by various means of enzyme immobilization. **Figure 16A** shows an amperometric calibration curve of a L-DOPA biosensor fabricated by immobilizing the PaMCO His396Ala/His459Ala enzyme with glutaraldehyde. **Figure 16B** shows an amperometric calibration curve of a L-DOPA biosensor fabricated by immobilizing the PaMCO His396Ala/His459Ala enzyme with 1, 2, and 3 mg/mL concentrations of PEDOT:PSS. **Figure 16C** shows an open circuit potential calibration curve of a L-DOPA biosensor fabricated by immobilizing the PaMCO His396Ala/His459Ala enzyme with copper sulfate nanoflowers. **Figure 16D** shows an amperometric calibration curve of a L-DOPA biosensor fabricated by immobilizing the PaMCO His396Ala/His459Ala enzyme with DSH-SAM. **Figure 16E** shows an amperometric calibration curve of a L-DOPA biosensor fabricated by immobilizing the PaMCO His396Ala/His459Ala enzyme with Nafion.

[0115] **Figure 17** shows a chronoamperometric response of the needle-type PaMCO His396Ala/His459Ala L-DOPA biosensor.

[0116] **Figure 18** shows an open circuit potential response of the needle-type PaMCO His396Ala/His459Ala L-DOPA biosensor sampled at different time points.

[0117] **Figures 19A-19C** show open circuit potential calibration curves for L-DOPA using wire working, counter, and reference electrodes for n=9 electrodes. **Figure 19A** shows the OCP curves vs time after pulsing a CA signal at 0.3 V for 180 seconds. **Figure 19B** shows the generated L-DOPA OCP calibration curve from 0.1 μ M up to 55 μ M taken at 240 second after the voltage pulse and the inset depicts the lower end of the concentration gradient ranging from 0.1 – 1.2 μ M

fit to a linear slope. **Figure 19C** replicates this protocol in human plasma, further confirming the potential of this biosensing scheme for L-DOPA detection in Parkinson's patients. The inset graph of **Figure 19C** shows the concentration gradient between 0-0.2 μM L-DOPA fit to a linear slope. The respective LOD was found to be 170 nM L-DOPA.

[0118] **Figures 20A-20C** show transient open circuit potential calibration curves for L-DOPA using wire working, counter, and reference electrodes for $n=9$ electrodes. **Figure 20A** shows the transient OCP curves vs time after pulsing a CA signal at 0.3 V for 180 seconds. **Figure 20B** shows the generated L-DOPA transient OCP calibration curve from 0.1 μM up to 55 μM taken at 240 second after the voltage pulse. **Figure 20C** replicates this protocol in human plasma, further confirming the potential of this biosensing scheme for L-DOPA detection in Parkinson's patients. The inset graph of **Figure 20C** shows the concentration gradient between 0-0.2 μM L-DOPA fit to a linear slope.

[0119] **Figures 21A-21B** show amperometric calibration curves for L-DOPA using wire working, counter, and reference electrodes for $n=9$ electrodes. **Figure 21A** shows the CA curves vs time whilst holding the voltage at 0.3 V vs Ag/AgCl reference. **Figure 21B** shows the generated L-DOPA calibration curve from 0.1 μM up to 55 μM taken at 3 seconds after the voltage pulse.

[0120] **Figures 22A-22D** show raw data from screen-printed single use sensor strips with various concentrations of immobilized enzyme at different L-DOPA concentration test points. Electrodes were prepared by drop casting the PaMCO His396Ala/His459Ala enzyme at concentrations of 0.1, 0.5, and 1 mg/mL. Open circuit potential measurements are shown at 5 μM of L-DOPA in **Figure 22A** and 20 μM of L-DOPA in **Figure 22B**. Amperometric measurements are shown at 5 μM of L-DOPA in **Figure 22C** and 20 μM of L-DOPA in **Figure 22D**.

[0121] **Figure 23** shows a schematic of the insertion of the pael888 gene encoding the PaMCO enzyme into a pET-11a expression vector.

[0122] **Figure 24A** shows the power output and **Figure 24B** shows the current density of an enzyme biofuel cell with a copper dehydrogenase anode paired with a PaMCO cathode.

[0123] **Figures 25A-25B** show measurements of L-DOPA obtained using a wire-type gold copper dehydrogenase sensor within a flow cell setup. **Figure 25A** shows the observed raw data, and **Figure 25B** shows reliable detection of L-DOPA in a concentration range of 0-55 μM in both increasing and decreasing concentrations.

[0124] **Figures 26A-26B** show an electrochemical protocol for a wire or needle type continuous levodopa sensor. **Figure 26A** shows the sensor protocol, alternating between chronoamperometry (CA), and open circuit potential (OCP), while calculating the real time transient open circuit potential. **Figure 26B** shows the ability to measure all three signals in parallel over the respective time course of detection, allowing for single point, intermittent, or continuous measurements.

[0125] **Figures 27A-27D** display the chronoamperometry, open circuit potential, and transient open circuit potential response to the addition of levodopa with the addition of an outer membrane of cellulose acetate at various weight to volume composition. **Figure 27A** displays the initial open circuit potential time course for outer membrane W/V ratios of 3, 5, and 7 percent. At 370 seconds 3 μ M levodopa was added. **Figure 27B** shows the open circuit potential timecourse for electrodes with and without pretreatment. Pretreatment was performed by dip coating the electrode into pure acetone for 5 seconds, followed by dipping the electrode into a 5% W/V cellulose acetate solution. At 370 seconds 3 μ M levodopa was added, showcasing a decrease in open circuit potential for both the pretreated and non-pretreated sensor. **Figure 27C** shows the open circuit potential response after pulsing the chronoamperometric signal of 0.3 V for 30 seconds for each of the outer membrane formulations, in 3 μ M levodopa solution. **Figure 27D** shows the transient open circuit response calculated from **Figure 27C** after the chronoamperometric pulse of 0.3V for 30 seconds.

[0126] **Figures 28A-28C** show the response to the addition of 25 μ M levodopa using open circuit potential for electrodes pretreated with 3 % (**Figure 28A**), 5 % (**Figure 28B**), and 7 % (**Figure 28C**) weight to volume of cellulose acetate as an outer membrane using three replicates. **Figure 28D** shows the percent change in signal upon the addition of 25 μ M levodopa for the different concentrations.

[0127] **Figures 29A-29B** show the response to the addition of 25 μ M levodopa using open circuit potential (**Figure 29A**) and chronoamperometry (**Figure 29B**) for 5 % W/V cellulose acetate outer membranes using different deposition methods with three replicates. Group 1 used a single dip coat for 5 seconds, followed by a 15 second dry period. Group 2 was dip coated twice for 5 seconds, followed by a 15 second dry period for each dip. For group 3 5 μ L of cellulose acetate solution was drop cast directly onto the electrode and allowed to dry in an incubator. Group

4 electrodes were pre-tested and calibrated, then dip coated 3 times for 5 seconds, followed by a 15 second dry period for each dip.

[0128] **Figure 30** depicts a L-DOPA sensor using a two electrode layout with 1 working electrode and 1 reference/counter electrode. The reference/counter electrode is wrapped around the working electrode, with $> 3X$ the total surface area. The working electrode is composed of insulated gold with the immobilized enzyme, and the reference/counter is composed of insulated silver with silver chloride. Single or multiple points of insulation may be removed from the working and reference/counter electrode.

[0129] **Figures 31A-C** show the L-DOPA sensor response in) human serum filtered with a 2 μ m filter using chronoamperometry (**Figure 31A**), open circuit potential (**Figure 31B**), and transient open circuit potential (**Figure 31C**). **Figure 31D-31F** show the limit of detection (LOD) and sensitivity of the sensor in both buffer and filtered human plasma.

[0130] **Figures 32A-32C** display chronoamperometric (**Figure 32A**), open circuit potential (**Figure 32B**), and transient open circuit potential (**Figure 32C**) calibration curves against L-DOPA. Standard error was calculated using 9 replicate microwire sensors.

[0131] **Figures 33A-33B** show signal changes prompted by the addition of endogenous and exogenous interferences. **Figure 33A** shows interferences selected considering their structural resemblance to levodopa, their role in the levodopa metabolic pathway, their co-administration with levodopa in Parkinson's treatment. **Figure 33B** shows common interference substrates for continuous glucose monitoring systems.

[0132] **Figures 34A-34C** show the 21-day storage stability of the L-DOPA sensor, depicting day 1, 7, and 21 levodopa calibration curves in 100 mM potassium phosphate buffer. The sensitivity was constant, remaining within 5% of the original response for all three metrics.

[0133] **Figures 35A-35D** show wires-type L-DOPA sensors evaluated using a flow cell, to both increase and decrease the L-DOPA concentration over the therapeutic range, and within expected time frames. The continuous monitoring of L-DOPA from 0.1 to 55 μ M over approximately 3 hours is shown, as well as the overlay of the increasing and decreasing calibration curves for open circuit potential (**Figures 35A-35B**) and transient open circuit potential (**Figure 35C-35D**).

[0134] **Figures 36A-36C** show time courses of both absolute levodopa concentration (**Figure 36A**), and rate of change in levodopa using a continuous levodopa sensor with two

peristaltic pumps (**Figure 36B**), by varying the concentration between 0 – 15 μM , and collecting data every 3 minutes. **Figure 36C** displays an enlarged view of both absolute and change in levodopa over time, demonstrating the feasibility of being able to “predict” changes in L-DOPA, leading to peaks or valleys in concentration. The curve of **Figure 36A** displays the real time change in L-DOPA, which enables the ability to “predict” or intervene prior to the onset of symptoms.

[0135] **Figure 37** shows calibration curves of an immobilized PaMCO compared to a PaMCO His396Ala/His459Ala mutant (CoDH) with and without oxygen. The CoDH shows little oxygen sensitivity and illustrates the effects of enzyme engineering to improve enzyme dehydrogenase activity for L-DOPA measurement.

[0136] **Figures 38A-38G** displays amperograms at six distinct applied potentials (0.2 V, 0.3 V, 0.4 V, 0.5 V, 0.6 V, and 0.7 V for **Figures 38A-38F**, respectively) with each individual graph displaying the electrochemical response at its respective applied potential. **Figure 38G** shows the calibration curves for all the applied potentials.

[0137] **Figure 39A-39I** show the impact of temperature, ionic concentration, and pH on sensor response. **Figures 39A, 39D, and 39G** illustrate the impacts of modulating 50-150 mM KCl on the chronoamperometric, open circuit potential, and transient open circuit potential signals, respectively, in a background of 10 μM levodopa solution. **Figures 39B, 39E, and 39H** display how changing the pH (6.0-8.0) of a 100 mM PPB solution affects the chronoamperometric, open circuit potential, and transient open circuit potential signals, respectively, when measuring 10 μM levodopa. **Figures 39C, 39F, and 39I** highlight the variations in the chronoamperometric, open circuit potential, and transient open circuit potential signals, respectively, when measuring L-DOPA concentrations of 1-55 μM in 100 mM PPB (pH 7.0) at room temperature versus body temperature.

[0138] **Figure 40A-40C** show the impact of applied voltage over the working electrode on various interfering compounds. **Figure 40A** demonstrates the chronoamperometric interference impact, **Figure 40B** represents the open circuit potential interference impact, and **Figure 40C** portrays the transient open circuit potential interference impact.

[0139] **Figures 41A-41F** display the impact of DSH concentration on chronoamperometric, open circuit potential, and transient open circuit potential signals at two potentials (0.3 and 0.4 V). The working electrode used was a DSH-CoDH GDE ($n=3$, $\Phi = 2$ mm), along with an Ag/AgCl junction electrode as the reference and a Pt electrode as the counter

electrode, across a concentration range of 0-55 μM levodopa in 100 mM PPB (pH 7.0). **Figure 41A** demonstrates the chronoamperometric signal, **Figure 41B** represents the OCP signal, and **Figure 41C** portrays the transient OCP signal from 100 μM DSH-CoDH GDEs at applied potentials of 0.3 V or 0.4 V. **Figures 41D, 41E, and 41F** illustrate the chronoamperometric signal, the OCP signal, and the transient OCP signal, respectively, from 500 μM DSH-CoDH GDEs at applied potentials of 0.3 V or 0.4 V. For chronoamperometric measurements, either a 0.3 V or 0.4 V overpotential was applied for 180 seconds, followed by immediate OCP measurement. The transient OCP was then determined by calculating the derivative of OCP with respect to time. Subsequent analysis, backed by LODs and sensitivities, revealed that a sensor configuration with 100 μM DSH at a 0.3 V potential provides a balance of enhancing chronoamperometric LOD while maintaining OCP and transient OCP LOD and sensitivity.

[0140] **Figures 42A-42C** show representative data serves to illustrate the typical structure of chronoamperometric, open circuit potential, and transient open circuit potential signals.

[0141] **Figure 43** shows the impact of the roughening process on the wire L-DOPA sensor's electrode surface area. The increase in surface area contributes to enhanced sensing performance of microwire levodopa sensors.

[0142] **Figures 44A-44C** show analysis of interferent effects on a copper dehydrogenase levodopa sensor as compared to a tyrosinase sensor. **Figure 44A**, a comparison of chronoamperometric interferent responses illustrates the sensor performance under constant potential conditions. **Figure 44B** displays the comparison of open circuit potential interferent responses, shedding light on sensor behavior without an applied external potential. **Figure 44C** presents the comparison of transient open circuit potential interferent responses. Across all employed electrochemical techniques, the engineered enzyme displayed significantly diminished interferent impact in comparison to tyrosinase.

[0143] **Figures 45A-45C** show the storage stability of the L-DOPA sensor over the first 7 days. **Figure 45A** displays the chronoamperometric calibration curves from day 1 to day 7, with an inset focusing on the calibration from 0-1.2 μM levodopa, to highlight the sensor's stability during the first week of storage. **Figure 45B** shows the open circuit potential calibration curves obtained over the same 7-day period. **Figure 45C** displays the transient open circuit potential calibration curves across the same 7-day period.

[0144] **Figure 46** shows the optimization for the wire type L-DOPA sensor using a variety of fabrication parameters, and measurement parameters.

[0145] **Figures 47A-47F** show square wave voltammetry of L-DOPA using PaMCO His396Ala/ His459Ala. **Figures 47A-47C** show raw Δ current response of 0 and 60 μ M L-DOPA at 1, 100 and 1000 mV/sec respectively. **Figures 47D** and **47E** shows sensitivity and correlation of Δ current to L-DOPA concentration at either 0.27 V or 0.7 V respectively. **Figure 47F** shows the calibration curve for Δ current vs L-DOPA at 0.27 V with a scan rate of 1mV/sec.

[0146] **Figures 48A-48G** show the calibration curves sensing of L-DOPA using PaMCO His396Ala/ His459Ala immobilized onto a gold disk electrode deposited with copper sulfide nanoflowers. **Figure 48A** shows the open circuit potential raw data response to increasing L-DOPA, **Figure 48B** shows the transient open circuit potential raw data, and **Figure 48C** shows the chronoamperometric raw data response. **Figure 48E** shows the open circuit potential calibration curve taken 5 seconds after pulsing 0.7 V vs Ag/AgCl, **Figure 48F** shows the transient open circuit potential calibration curve, each point taken 1 second after pulsing 0.7 V, and **Figure 48G** shows the chronoamperometric calibration curve, each point is a 5 second average measured at steady state.

[0147] **Figure 49** shows the structure of PaMCO F290I mutant (PDB ID: 6K3D) and the location of histidine residues related to T2/T3 Cu coordination in the PaMCO mutant.

[0148] **Figure 50** shows the spectrum analysis of the cofactor in copper dehydrogenases derived from *Pyrobaculum aerophilum* MCO (PaMCO).

[0149] **Figure 51** shows the alignment of MCO homologues with T1 and T2/T3 ligands indicated.

[0150] **Figures 52A-52C** show the characterization of the oxidase activity of *Escherichia coli* multicopper oxidase (CueO) wild type (WT) and mutants.

[0151] **Figure 53** shows the spectrum analysis of the cofactor in CueO-His mutants.

[0152] **Figure 54** shows the SDS-PAGE analyses of purified *Bacillus subtilis* outer spore coat copper-dependent laccase, CotA wild type and mutant His422Ala.

[0153] **Figure 55** shows the relative oxidase activities of *Bacillus subtilis* outer spore coat copper-dependent laccase, CotA wild type and mutant His422Ala.

DETAILED DESCRIPTION

[0154] Currently, Parkinson's Disease (PD) treatment is hindered by a lack of technology for the detection of L-DOPA. Providing a proper therapeutic dose is challenging without data, and both under-administering and over-administering L-DOPA has serious side effects which effect patients' quality of life. There are no L-DOPA sensors available on the market, and no practical L-DOPA sensors have been reported in scientific literature. While there have been several attempts to develop either a L-DOPA point of care sensor, or continuous platform,^{6,7,12} challenges with enzyme substrate specificity, stability, and biosensor performance have prevented the development of commercially available L-DOPA sensors. Specifically, the development of commercial products has been limited by the fact that majority of reported L-DOPA sensors utilize enzymes such as tyrosinase which are not specific towards L-DOPA. Disclosed herein is a biosensor which utilizes a novel copper dehydrogenase enzyme, engineered from a multicopper oxidase enzyme to specifically detect L-DOPA levels, addressing this longstanding need in the field of Parkinson's treatment. Continuous L-DOPA biosensors, point-of-care testing (POCT), and personal use L-DOPA monitoring systems disclosed herein have the potential to significantly enhance the management of stage II PD and minimize "off-time" PD symptoms.

[0155] The presently disclosed subject matter will now be described more fully hereinafter. However, many modifications and other embodiments of the presently disclosed subject matter set forth herein will come to mind to one skilled in the art to which the presently disclosed subject matter pertains having the benefit of the teachings presented in the foregoing descriptions. Therefore, it is to be understood that the presently disclosed subject matter is not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims. In other words, the subject matter described herein covers all alternatives, modifications, and equivalents. In the event that one or more of the incorporated literature, patents, and similar materials differs from or contradicts this application, including but not limited to defined terms, term usage, described techniques, or the like, this application controls. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in this field. All publications, patent applications, patents and other references mentioned herein are incorporated by reference into their entirety.

I. Overview

[0156] Multicopper oxidases (MCOs) belong to a protein superfamily of enzymes which catalyze the four-electron reduction of oxygen to water by the oxidation of various substrates. They catalyze the one-electron oxidation of a broad range of compounds including substituted phenols, arylamines and aromatic thiols to the corresponding radicals as shown in **Figure 1**. MCOs contain at least four copper atoms, which are classified as type 1 (T1), type 2 (T2) or type 3 (T3)^{1,2,3}. The oxidation of a substrate starts at a T1 mononuclear copper center, then electrons are transferred internally to the trinuclear copper center T2/T3 where oxygen (O₂) is reduced by four electrons, yielding two water molecules. The Satomura research group recently found a novel and hyperthermostable MCO derived from an hyperthermophilic archaeon *Pyrobaculum aerophilum*, elucidated its structure⁴ and engineered the enzyme⁵ to increase its catalytic activity suitable for bioelectrochemical applications. Multicopper oxidases derived from *Pyrobaculum aerophilum*, comprise two copper atoms at the T3 site which are coordinated by His94, His132, His134, His396, His459, and His461 residues and a copper at the T2 site coordinated by His92 and His394 as copper atom ligands.⁴

[0157] Native (e.g., wild-type) MCOs use oxygen as an electron acceptor. *See Figure 1*. However, for certain biosensing applications, such as monitoring the presence of substituted phenols, arylamines and aromatic thiols, the reaction with oxygen is always competing with the electron transfer to synthetic electron acceptors. Moreover, MCOs are capable of direct electron transfer to the electrode when they are immobilized on the electrode. However, the activity toward oxygen does not allow for the use of an electrode for the oxidative half reaction to oxidize and monitor substrates which react via the reductive half reaction. Therefore, aspects of the disclosure relate to further engineering MCOs to have reduced oxidase activity towards oxygen to improve enzymatic properties for biosensing applications. The resulting enzymes have reduced oxidase ability but retain dehydrogenase capabilities and herein are referred to as copper dehydrogenases (CoDh). Without wishing to be bound by theory, it is believed that the copper dehydrogenases may no longer comprise multiple copper atoms and may only comprise a single T1 copper atom.

[0158] In embodiments, provided herein are amino acid sequences of engineered copper dehydrogenases, such those derived from *Pyrobaculum aerophilum* multicopper oxidase (EC 1.10.3.2). In embodiments, provided herein are methods of making engineered copper dehydrogenases with reduced oxidase activity towards oxygen and their application as biosensors,

including but not limited to electrochemical L-DOPA sensors. Shown herein, engineered copper dehydrogenases have repressed oxidase activity towards oxygen and can have increased catalytic activity for a substrate, such as L-DOPA, compared to wild-type MCOs. For example, provided herein are engineered copper dehydrogenases with oxidase activity towards oxygen that is repressed more than 50%, or 60%, or 70%, or 80%, or 90%, or 91%, or 92%, or 93%, or 94%, or 95%, or 96%, or 97%, or 98%, or 99%, or 99.5, or 99.9% compared to MCOs without the mutations provided herein.

[0159] It should be known that the multicopper oxidase and copper dehydrogenase enzymes disclosed herein may or may not include an initial signal peptide sequence comprising a cytoplasmic domain and transmembrane helix, which is found at the N-terminus of wild-type multicopper oxidase sequences and is typically 28 amino acid residues in length. Mutations are herein described by their numbered position in the entire enzyme sequence, which includes the signal peptide sequence (e.g., His94Ala, His 132Ala, Phe290Ile, His396Ala, and His459Ala). However, in certain embodiments provided herein, enzymes were engineered and produced without the initial signal peptide sequence. Therefore, mutations of amino acid residues of multicopper oxidases and copper dehydrogenases disclosed herein may be referred to using numbered positions different from the actual location of the mutation in the disclosed sequence, due to the lack of inclusion of the signal peptide. For example, the mutation referred to as His94Ala is at position 66 of SEQ ID NO:7, as the first 28 amino acid residues comprising the initial signal peptide are not included in SEQ ID NO:7.

[0160] In some embodiments, provided herein, engineered copper dehydrogenases are derived from *Pyrobaculum aerophilum* multicopper oxidase, wherein the wild-type enzyme has an amino acid sequence set forth in SEQ ID NO: 8.

[0161] *Wild-type multicopper oxidase from Pyrobaculum aerophilum* without signal sequence + Methionine at position 1 (UniProtID: Q8ZWA8)

```
MTGEVKRPE TSAPVPPLIKEATYIEATASGYMAEGVLNPTIILRRGQRVDMTLKN
KLTEPTIVHWHGFDVNWHNDAHPSFAITPGESYNYSFDVVNRAGTYLYHPHPHG
LTAKQFYMGQLGLVIVEDSGSDLGFKYGVNDLPLVISDRRFIGGAPVYNPTPME
MIAGFLGNAVLVNGVKDAVFKLSGGSYRLRLVNGSNARLYMLSIVKKNGDVVP
MRLIAVDQGFLARPIEVRLFLAPAERAEEVVVELGEGVYLLKNTPFDPMHLEMG
HGMQEALPEGSEYTIATFLVEGKGAEVPVEALSDPPPEPPKPTRTRRFALSLSGM
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QWTINGMFWNASNPLFEHVSVEGVELWEIVNDKASMPHPMHLHGFPMWIIERK
 DSPRQVAELAVDNRGRLPTDLGLKDTVLIWPGETVKIVVNFDAAKKRGQLFPFHC
 HNLEHEDGGMMINIAVK (SEQ ID NO: 8)

[0162] In some embodiments, the engineered copper dehydrogenase further comprises a signal sequence (i.e., at the N-terminus). In some embodiments, the signal sequence has an amino acid sequence set forth in SEQ ID NO: 18. In such embodiments, the initial methionine provided for peptide sequences without the signal sequence (e.g., position 1 of SEQ ID NOs: 1-8) may not be included in the amino acid sequence comprising the signal sequence.

[0163] *Signal sequence for multicopper oxidase from Pyrobaculum aerophilum*
 MITRRRFLQIGLGAGAMLAMGFTLQYILR (SEQ ID NO: 18)

[0164] In embodiments, disclosed herein, engineered copper dehydrogenases derived from *Pyrobaculum aerophilum* harbor one or more mutations to improve catalytic activity, such as Phe290Ile. In embodiments, the Phe290Ile mutation to improve catalytic activity corresponds to position 262 of the amino acid sequence set forth in SEQ ID NO: 7.

[0165] *Multicopper oxidase from Pyrobaculum aerophilum with Phe290Ile* without signal sequence + Methionine at position 1 (UniProtID: Q8ZWA8)

MTGEVKRPETSAPVPPLIKEATYIEATASGYMAEGVLNPTIILRRGQRVDMTLKN
 KLTEPTIVHWHGFDVNWHNDAHPSFAITPGESYNYSFDVVNRAGTYLYHPHPHG
 LTAKQFYMGQLGLVIVEDSGSDLGFKYGVNDLPLVISDRRFIGGAPVYNPTPME
 MIAGFLGNAVLVNGVKDAVFKLSGGSYRLRLVNGSNARLYMLSIVKKNGDVVP
 MRLIAVDQGFLARPIEVRALFLAPAERAEEVVVELGEGVYLLKNTPIDPMHLEMG
 HGMQEALPEGSEYTIATFLVEGKGEAVPVEALSDPPPEPPKPTRTRRFALSLSGM
 QWTINGMFWNASNPLFEHVSVEGVELWEIVNDKASMPHPMHLHGFPMWIIERK
 DSPRQVAELAVDNRGRLPTDLGLKDTVLIWPGETVKIVVNFDAAKKRGQLFPFHC
 HNLEHEDGGMMINIAVK (SEQ ID NO: 7)

[0166] In embodiments, provided herein are various engineered copper dehydrogenases harboring mutations in type 2 and/or type 3 copper atom ligands, which results in repressed oxidase activity towards oxygen while preserving direct electron transfer (DET) ability. In embodiments, an amino acid residue comprising a type 2 and/or type 3 copper atom ligand is substituted to a different amino acid residue. In some embodiments, an amino acid residue comprising a type 2

and/or type 3 copper atom ligand is substituted from a histidine residue to another amino acid residue. In some embodiments, an amino acid residue comprising a type 2 and/or type 3 copper atom ligand is substituted from a histidine residue to an alanine residue.

[0167] In embodiments, disclosed herein, engineered copper dehydrogenases derived from *Pyrobaculum aerophilum* can have one or more mutations of the type 2 and/or type 3 copper atom ligands selected from the group His92, His94, His132, His134, His394, His396, His459, and His461, which correspond to positions 64, 66, 104, 106, 366, 368, 431, and 433 of SEQ ID NO: 7, respectively. In some embodiments, the engineered copper dehydrogenase derived from *Pyrobaculum aerophilum* has one or more mutations of the type 2 and/or type 3 copper atom ligands selected from the group His92Ala, His94Ala, His132Ala, His134Ala, His394Ala, His396Ala, His459Ala, and His461Ala. Figure 49 shows the location of His94, His134, His396, and His459 as copper atom ligands in *Pyrobaculum aerophilum* multicopper oxidase.

[0168] In one embodiment, the engineered copper dehydrogenase derived from *Pyrobaculum aerophilum* harbors a Phe290Ile mutation to increase catalytic activity and a His94Ala mutation to reduce oxidase activity towards oxygen. The Phe290Ile mutation corresponds to position 262 and the His94Ala mutation corresponds to position 66 of the amino acid sequence set forth in SEQ ID NO: 7. In one embodiment, the engineered copper dehydrogenase comprises the amino acid sequence set forth in SEQ ID NO: 1.

[0169] *Copper dehydrogenase derived from Pyrobaculum aerophilum with His94Ala/Phe290Ile without signal sequence + Methionine at position 1 (UniProtID: Q8ZWA8)*

MTGEVKRPETSAPVPPLIKEATYIEATASGYMAEGVLNPTIILRRGQRVDMTLKN
KLTEPTIVHWAGFDVNWHNDAHPSFAITPGESYNYSFDVVNRAGTYLYHPPHGH
LTAKQFYMGQLGLVIVEDSGSDLGFKYGVNDLPLVISDRRFIIGGAPVYNPTPME
MIAGFLGNAVLVNGVKDAVFKLSGGSYRLRLVNGSNARLYMLSIVKKNGDVVP
MRLIAVDQGFLARPIEVRALFLAPAERAEEVVVELGEGVYLLKNTPIDPMHLEMG
HGMQEALPEGSEYTIATFLVEGKGEAVPVEALSDPPPEPPKPTRTRRFALSLSGM
QWTINGMFWNASNPLFEHVSVEGVELWEIVNDKASMPHPMHLHGFPMWIIERK
DSPRQVAELAVDNRGRLPTDLGLKDTVLIWPGETVKIVVNFDKAKRGQLFPFHC
HNLEHEDGGMMINIAVK (SEQ ID NO: 1)

[0170] In another embodiment, the engineered copper dehydrogenase derived from *Pyrobaculum aerophilum* harbors a Phe290Ile mutation to increase catalytic activity and a

His132Ala mutation to reduce oxidase activity towards oxygen. The Phe290Ile mutation corresponds to position 262 and the His132Ala mutation corresponds to position 104 of the amino acid sequence set forth in SEQ ID NO: 7. In one embodiment, the engineered copper dehydrogenase comprises the amino acid sequence set forth in SEQ ID NO: 2.

[0171] *Copper dehydrogenase derived from Pyrobaculum aerophilum with His132Ala/Phe290Ile* without signal sequence + Methionine at position 1 (UniProtID: Q8ZWA8)

MTGEVKRPETSAPVPPLIKEATYIEATASGYMAEGVLNPTIILRRGQRVDMTLKN
 KLTEPTIVHWHGFDVNWHNDAHPSFAITPGESYNYSFDVVNRAGTYLYAPHPHG
 LTAKQFYMGQLGLVIVEDSGSDLGFKYGVNDLPLVISDRRFIIGGAPVYNPTPME
 MIAGFLGNAVLVNGVKDAVFKLSGGSYRLRLVNGSNARLYMLSIVKKNGDVVP
 MRLIAVDQGFLARPIEVRALFLAPAERAEEVVVELGEGVYLLKNTPIDPMHLEMG
 HGMQEALPEGSEYTIATFLVEGKGEAVPVEALSDPPPEPPKPTRTRRFALSLSGM
 QWTINGMFWNASNPLFEHVSVEGVELWEIVNDKASMPHPMHLHGFPMWIIERK
 DSPRQVAELAVDNRGRLPTDLGLKDTVLIWPGETVKIVVNFDAAKKRGQLFPFHC
 HNLEHEDGGMMINIAVK (SEQ ID NO: 2)

[0172] In one embodiment, the engineered copper dehydrogenase derived from *Pyrobaculum aerophilum* harbors a Phe290Ile mutation to increase catalytic activity and a His396Ala mutation to reduce oxidase activity towards oxygen. The Phe290Ile mutation corresponds to position 262 and the His396Ala mutation corresponds to position 368 of the amino acid sequence set forth in SEQ ID NO: 7. In one embodiment, the engineered copper dehydrogenase comprises the amino acid sequence set forth in SEQ ID NO: 3.

[0173] *Copper dehydrogenase derived from Pyrobaculum aerophilum with Phe290Ile/His396Ala* without signal sequence + Methionine at position 1 (UniProtID: Q8ZWA8)

MTGEVKRPETSAPVPPLIKEATYIEATASGYMAEGVLNPTIILRRGQRVDMTLKN
 KLTEPTIVHWHGFDVNWHNDAHPSFAITPGESYNYSFDVVNRAGTYLYHPPHPG
 LTAKQFYMGQLGLVIVEDSGSDLGFKYGVNDLPLVISDRRFIIGGAPVYNPTPME
 MIAGFLGNAVLVNGVKDAVFKLSGGSYRLRLVNGSNARLYMLSIVKKNGDVVP
 MRLIAVDQGFLARPIEVRALFLAPAERAEEVVVELGEGVYLLKNTPIDPMHLEMG
 HGMQEALPEGSEYTIATFLVEGKGEAVPVEALSDPPPEPPKPTRTRRFALSLSGM
 QWTINGMFWNASNPLFEHVSVEGVELWEIVNDKASMPHPMHLAGFPMWIIERK

DSPRQVAELAVDNRGRLPTDLGLKDTVLIWPGETVKIVVNFDAAKRGQLFPFHC
HNLEHEDGGMMINIAVK (SEQ ID NO: 3)

[0174] In one embodiment, the engineered copper dehydrogenase derived from *Pyrobaculum aerophilum* harbors a Phe290Ile mutation to increase catalytic activity and a His459Ala mutation to reduce oxidase activity towards oxygen. The Phe290Ile mutation corresponds to position 262 and the His459Ala mutation corresponds to position 431 of the amino acid sequence set forth in SEQ ID NO: 7. In one embodiment, the engineered copper dehydrogenase comprises the amino acid sequence set forth in SEQ ID NO: 4.

[0175] *Copper dehydrogenase derived from Pyrobaculum aerophilum with Phe290Ile/His459Ala* without signal sequence + Methionine at position 1 (UniProtID: Q8ZWA8)

MTGEVVRPETSAPVPLIKEATYIEATASGYMAEGVLNPTIILRRGQRVDMTLKN
KLTEPTIVHWHGFDVNWHNDAHPSFAITPGESYNYSFDVVNRAGTYLYHPHPHG
LTAKQFYMGQLGLVIVEDSGSDLGFKYGVNDLPLVISDRRFIIGAPVYNPTPME
MIAGFLGNAVLVNGVKDAVFKLSGGSYRLRLVNGSNARLYMLSIVKKNGDVVP
MRLIAVDQGFARPIEVRALFLAPAERAEEVVVELGEGVYLLKNTPIDPMHLEMG
HGMQEALPEGSEYTIATFLVEGKGEAVPVEALSDPPPEPPKPTRTRRFALSLSGM
QWTINGMFWNASNPLFEHVSVEGVLEWIVNDKASMPHPMHLHGFPMWIIEK
DSPRQVAELAVDNRGRLPTDLGLKDTVLIWPGETVKIVVNFDAAKRGQLFPFAC
HNLEHEDGGMMINIAVK (SEQ ID NO: 4)

[0176] In another embodiment, the engineered copper dehydrogenase derived from *Pyrobaculum aerophilum* harbors a Phe290Ile mutation to increase catalytic activity and His396Ala/His459Ala mutations to reduce oxidase activity towards oxygen. The Phe290Ile mutation corresponds to position 262, the His396Ala mutation corresponds to position 368, and the His459Ala mutation corresponds to position 431 of the amino acid sequence set forth in SEQ ID NO: 7. In one embodiment, the engineered copper dehydrogenase comprises the amino acid sequence set forth in SEQ ID NO: 5.

[0177] *Copper dehydrogenase derived from Pyrobaculum aerophilum with Phe290Ile/His396Ala/His459Ala* without signal sequence + Methionine at position 1 (UniProtID: Q8ZWA8)

MTGEVVRPETSAPVPLIKEATYIEATASGYMAEGVLNPTIILRRGQRVDMTLKN
KLTEPTIVHWHGFDVNWHNDAHPSFAITPGESYNYSFDVVNRAGTYLYHPHPHG

LTAKQFYMGQLGLVIVEDSGSDLGFKYGVNDLPLVISDRRFI GGAPVYNPTPME
 MIAGFLGNAVLVNGVKDAVFKLSGGSYRLRLVNGSNARLYMLSIVKKNGDVVP
 MRLIAVDQGFLARPIEVRALFLAPAERA EVVVELGEGVYLLKNTPIDPMHLEMG
 HGMQEALPEGSEYTIATFLVEGKGEAVPVEALSDPPPEPPKPTRTRRFALSLSGM
 QWTINGMFWNASNPLFEHVSVEGVELWEIVNDKASMPHPMHLAGFPMWIIERK
 DSPRQVAELAVDNRGRLPTDLGLKDTVLIWPGETVKIVVNFD AKKRGQLFPFAC
 HNLEHEDGGMMINIAVK (SEQ ID NO: 5)

[0178] In another embodiment, the engineered copper dehydrogenase derived from *Pyrobaculum aerophilum* harbors a Phe290Ile mutation to increase catalytic activity and His94Ala/His396Ala/His459Ala mutations to reduce oxidase activity toward oxygen. The Phe290Ile mutation corresponds to position 262, the His94Ala mutation corresponds to position 66, the His396Ala mutation corresponds to position 368, and the His459Ala mutation corresponds to position 431 of the amino acid sequence set forth in SEQ ID NO: 7. In one embodiment, the engineered copper dehydrogenase comprises the amino acid sequence set forth in SEQ ID NO: 6.

[0179] *Copper dehydrogenase derived from Pyrobaculum aerophilum with His94Ala/Phe290Ile/His396Ala/His459Ala* without signal sequence + Methionine at position 1 (UniProtID: Q8ZWA8)

MTGEVKRPETSAPVPPLIKEATYIEATASGYMAEGVLNPTIILRRGQRVDMTLKN
 KLTEPTIVHWAGFDVNWHNDAHPSFAITPGESYNYSFDVVNRAGTYLYHPPHGH
 LTAKQFYMGQLGLVIVEDSGSDLGFKYGVNDLPLVISDRRFI GGAPVYNPTPME
 MIAGFLGNAVLVNGVKDAVFKLSGGSYRLRLVNGSNARLYMLSIVKKNGDVVP
 MRLIAVDQGFLARPIEVRALFLAPAERA EVVVELGEGVYLLKNTPIDPMHLEMG
 HGMQEALPEGSEYTIATFLVEGKGEAVPVEALSDPPPEPPKPTRTRRFALSLSGM
 QWTINGMFWNASNPLFEHVSVEGVELWEIVNDKASMPHPMHLAGFPMWIIERK
 DSPRQVAELAVDNRGRLPTDLGLKDTVLIWPGETVKIVVNFD AKKRGQLFPFAC
 HNLEHEDGGMMINIAVK (SEQ ID NO: 6)

[0180] In embodiments, a device is provided for assaying L-DOPA in a sample, where the device includes an engineered copper dehydrogenase with reduced oxidase activity towards oxygen as described herein. In some instances, an enzyme electrode is provided, where the enzyme electrode includes an engineered copper dehydrogenase as described herein that is immobilized on the electrode. In other embodiments, an enzyme sensor is provided for assaying L-DOPA, where

the enzyme sensor includes an enzyme electrode as described herein as a working electrode. In another embodiment, a kit is provided for assaying L-DOPA in a sample, where the kit includes an engineered copper dehydrogenase as described herein.

[0181] Provided herein are engineered copper dehydrogenases with drastically reduced oxidase activity towards oxygen and enhanced dehydrogenase activity, including towards L-DOPA. In some embodiments, the engineered copper dehydrogenases are selected from the group consisting of *Pyrobaculum aerophilum* derived multicopper oxidase, laccase, bilirubin oxidase and tyrosinase. These engineered copper dehydrogenases are created by introducing mutations to copper atom ligands to repress oxidase activity towards oxygen while maintaining direct electron transfer (DET) capabilities to an electrode.

[0182] In some embodiments, the engineered copper dehydrogenases, such as the described *Pyrobaculum aerophilum* derived multicopper oxidases, are highly specific and selective for L-DOPA as a substrate and do not react with other physiologically relevant compounds including but not limited to quinones, sugars, phenols, and or C-DOPA. These engineered copper dehydrogenases are useful as an enzyme in a L-DOPA monitoring kit, biomolecular recognition element of electrochemical biosensors such as for L-DOPA monitoring, and for implantable or wearable sensors for continuous L-DOPA monitoring. Provided herein are engineered copper dehydrogenases suitable for L-DOPA biosensing.

[0183] In some embodiments, phenolic compounds other than L-DOPA can be substrates for the engineered copper dehydrogenases. In some embodiments, the substrate is a phenolic compound derived from lignin, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 2,6-dimethoxyphenol, 4-methoxyphenol, phenol, hydroquinone, 2,4-dichlorophenol, p-benzoquinone, o-benzenediol, m-benzenediol, or p-benzenediol.

[0184] In some embodiments, the engineered copper dehydrogenase is selected from the group consisting of a *Escherichia coli* derived multicopper oxidase (CueO), a *Bacillus subtilis* derived laccase (CotA), a *Thermus thermophilus* derived laccase (TTMCO), a *Myrothecium verrucaria* derived bilirubin oxidase (MyBOD), a *Bacillus pumilus* derived laccase, a *Bacillus licheniformis* derived laccase, a *Streptomyces cyaneus* derived laccase, and a *Streptomyces lavendulae* derived laccase. The positions of the corresponding histidine residues that are type 2 and type 3 copper atom ligands for different species of multicopper oxidase enzymes are shown in Table 2. An alignment of the homologous sequences indicating the residues coordinating the T1,

T2, and T3 copper atoms is shown in **Figure 51**. Engineered copper dehydrogenase enzymes can be produced by mutating one or more (or all) of the histidine residues coordinating the T2/T3 coppers in the provided positions (i.e. to a non-coordinating amino acid such but not limited to alanine or valine) to repress oxidase activity of the enzymes and promote DET to an electrode.

[0185] Table 2. T2 and T3 ligands of various multicopper oxidase enzymes.

	SEQ ID NO	T2 ligands		T3 ligands					
PaMCO	8	64	366	66	104	106	368	431	433
CueO	19	101	446	103	141	143	448	498	500
CotA	20	105	422	107	153	155	424	491	493
TTMCO	21	95	400	97	135	137	402	449	451
MyBOD	22	132	439	134	171	173	441	494	496
BpLac	23	103	422	105	151	153	424	491	493
BiLac	24	103	421	105	151	153	423	490	492
ScLac	25	145	511	147	185	187	513	586	588
SiLac	26	145	514	147	185	187	516	589	591

[0186] In some embodiments, provided herein, engineered copper dehydrogenases are derived from *Escherichia coli* multicopper oxidase (WP_166488460, UniProtID:P36649, CueO). The wild-type enzyme *Escherichia coli* multicopper oxidase has an amino acid sequence set forth in SEQ ID NO: 19. In some embodiments, the copper dehydrogenase comprises a sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 99.9% sequence identity to any one of SEQ ID NOs:19, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 101, 103, 141, 143, 446, 448, 498 and 500 of SEQ ID NO: 19 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 19.

[0187] *Wild-type multicopper oxidase from Escherichia coli* multicopper oxidase, WP_166488460, UniProtID:P36649, CueO

MQRRDFLKYSVALGVASALPLWSRAVFAAERPTLPIPDLLTTDARNRIQLTIGAG
 QSTFGGKTATTWGYNGNLLGPAVKLQRGKAVTVDIYNQLTEETTLHWHGLEVP
 GEVDGGPQGIIPPGGKRSVTLNVDQPAATCWFHPHQHGKTGRQVAMGLAGLVV
 IEDDEILKLMLPKQWGIDDPVIVQDKKFSADGQIDYQLDVMTAAVGWFGDTLL
 TNGAIYPQHAAPRGWLRLRLLLNGCNARSLNFATSDNRPLYVIASDGGLPEPVK
 VSELPVLMGERFEVLVEVNDNKPFDLVTLPVSQMGMAIAPFDKPHPVMRIQPIAI
 SASGALPDTLSSLPALPSLEGLTVRKLQLSMDPMLDMMGMQMLMEKYGDQAM
 AGMDHSQMMGHMGMHGNMNMNHGGKFDFFHHANKINGQAFDMNKPMFAAA
 KGQYERWVISGVGDMMLHPFHIIHGTQFRILSENGKPPAAHRAGWKDTVKEGNN
 VSEVLVKFNHDAPKEHAYMAHCHLLEHEDTGMMLGFTV(SEQ ID NO: 19)

[0188] In some embodiments, provided herein, engineered copper dehydrogenases are derived from *Bacillus subtilis* outer spore coat copper-dependent laccase (WP_106610907, UniProtID:P07788, CotA). The wild-type *Bacillus subtilis* laccase enzyme has an amino acid sequence set forth in SEQ ID NO:20. In some embodiments, the copper dehydrogenase comprises a sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 99.9% sequence identity to SEQ ID NO:20, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 105, 107, 153, 155, 422, 424, 491 and 493 of SEQ ID NO: 20 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 20.

[0189] *Wild-type multicopper oxidase from Bacillus subtilis* outer spore coat copper-dependent laccase (multicopper oxidase), WP_106610907, UniProtID:P07788, CotA

MTLEKFVDALPIPDTLKPVQQSKEKTYEVTMEECTHQLHRDLPPTRLWGYNGL
 FPGPTIEVKRNENVYVKWMNNLPSTHFLPIDHTIIHSDSQHEEPEVKTVVHLHGG
 VTPDDSDGYPEAWFSKDFEQTPYFKREVYHYPNQQRGAILWYHDHAMALTRL
 NVYAGLVGAYIIHDPKEKRLKLPSDEYDVPLLITDRITINEDGSLFYPSAPENPSPSL
 PNPSIVPAFCGETILVNGKVWPYLEVEPRKYRFRVINASNTRTYNLSLDNGGDFIQ
 IGSDGGLLPRSVKLNSFSLAPAERYDIIIDFTAYEGESIILANSAGCGGDVNPETDA
 NIMQFRVTKPLAQKDESRKPKYLASYPSVQHERIQNIRTLKLAGTQDEYGRPVL
 LNNKRWHDPVTETPKVGTTEIWSIINPTRGTHPIHLHLVSFRVLDRRPFDIARYQE

SGELSYTGPAVPPPPSEKGWKDTIQAHAGEVLRIAATFGPYSGRYVWHCHILEHE
DYDMMRPMDITDPHK (SEQ ID NO: 20)

[0190] In some embodiments, provided herein, engineered copper dehydrogenases are derived from *Thermus thermophilus* laccase (TTMCO, WP_011173754.1, UniProtID: F6DF14). The wild-type *Thermus thermophilus* laccase enzyme has an amino acid sequence set forth in SEQ ID NO:21. In some embodiments, the copper dehydrogenase comprises a sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 99.9% sequence identity to SEQ ID NO:21, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 95, 97, 135, 137, 400, 402, 449 and 451 of SEQ ID NO: 21 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 21.

[0191] *Wild-type multicopper oxidase from Thermus thermophilus* laccase (a multicopper oxidase), WP_011173754.1, UniProtID: F6DF14

MNTDRRTLLKLTAGLLLSPLARGQASFPEPPVLKSREGLLEVRLKAAPTPTVTVAG
REARLWTYGGSFPGPTLRVRPGDTRLELENLLPESTNLHWHGLPISPKVDDPFL
EIPPRETWSYVFTVPQDLAGTFWYHPHLHGRVAPQLFAGLAGAIVVESPVGDIPE
LREAEEHLLVLKDLELASGRPAAHTPMDWINGKEGNLLL VNGASRPTLRASKAT
LRLRLNASNARYYRLQLEGHPLYLIASDGGFLEEPYEVPELLLAPGERAEVLVR
FQKEGAFRLLALPYDRGVHMMGGMMEHMGHGGMAMGTSQRPQTLLTLVAPPRP
KPLPLPKALAKLPALSPNQARVTRRITFTEDMMAGRFFINGKTFDHRRVDFRGRV
GDLEVWELNQGDMDHPFHLHTHPFQVLSVNGKAFFPYRALKDVVNLKAKEVV
RLLVPLKDLPGKTVFHCHIVEHEDRGMMGVLEVG (SEQ ID NO: 21)

[0192] In some embodiments, provided herein, engineered copper dehydrogenases are derived from *Myrothecium verrucaria* bilirubin oxidase (MyBOD, UniProtID:Q12737). The wild-type *Myrothecium verrucaria* bilirubin oxidase enzyme has an amino acid sequence set forth in SEQ ID NO:22. In some embodiments, the copper dehydrogenase comprises a sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 99.9% sequence identity to SEQ ID NO:22, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 132, 134, 171, 173, 439, 441, 494 and

496 of SEQ ID NO: 22 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 22.

[0193] *Wild-type multicopper oxidase from Myrothecium verrucaria Bilirubin oxidase (a multi copper oxidase), UniProtID:Q12737*

MFKHTLGAAALSLLFNSNAVQASVPETSPATGHLFKRVAQISPQYPMFTVPLPIP
PVKQPRLTVTNPVNGQEIWYYEVEIKPFTHQVYPDLGSADLVGYDGMSPGPTFQ
VPRGVETVVRFINNAEAPNSVHLHGFSFRAAFDGAEDITEPGSFKDYYYPNRQ
SARTLWYHDHAMHITAENAYRGQAGLYMLTDPaedALNLPSGYGEFDIPMILTS
KQYTANGNLVTTNGELNSFWGDVIHVNGQPWPfKNVEPRKYRFRFLDAAVSRS
FGLYFADTDaidTRLpFKVIASDSGLLEHPADTSLLYISMAERYEVVDFDSDYAG
KTIELRNLGGSIGGIGTDTDYDNTDKVMRFVVADDTTQPDTSVVPANLRDVPFPS
PTTNTPRQFRFGRTGPTWTINGVAFADVQNRLLANVPVGTVERWELINAGNGWT
HPIHIHLVDFKVISRTSGNNARTVMpyESGLKDvVWLGRRETvvVEAHYAPFPG
VYMFHCHNLIHEDHDMMAAFNATVLPDYGYNATVFVDPMEELWQARPYELGE
FQAQSGQFSVQAVTERIQTMAEYRPYAAADE (SEQ ID NO: 22)

[0194] In some embodiments, provided herein, engineered copper dehydrogenases are derived from BpLac: *Bacillus pumilus* laccase (UniProtID:I3RYX9). The wild-type *Bacillus pumilus* laccase enzyme has an amino acid sequence set forth in SEQ ID NO:23. In some embodiments, the copper dehydrogenase comprises a sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 99.9% sequence identity to SEQ ID NO:23, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 103, 105, 151, 153, 422, 424, 491 and 493 of SEQ ID NO: 23 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 23.

[0195] *Wild-type multicopper oxidase from BpLac: Bacillus pumilus Laccase, UniProtID:I3RYX9 (a multicopper oxidase)*

MNLEKFVDELPIPEVAEPVKKNPRQTYYEIAMEEVFLKVHRDLpPTKLWTYNGS
LPGPTIHANRNEKVKVKWMNKLPLKHFLpVDHTIHEGHHDEPEVKTVVHLHGG
VTPASSDGYPEAWFSRDfEATGPFFEREVYEYPNHQQACTLWYHDHAMALTRL
NVYAGLAGFYLIStDAFEKSLELPKDDYDIPLMIMDRtFQEDGSLFYPSRPNDTPE

DSDIPDPSIVPFFCGETILVNGKVWPYLEVEPRKYRFRILNASNTRTYELHLDNDA
TIMQIGSDGGFLPRPVHRHQSFSIAPAERFDVIIDFSAYENKTITLKNTAGCGQDVNP
ETDANIMQFKVTRPLKGRVPKTLRPIFKPLPPLRPSRADRERTLTLTGTQDKYGRP
ILLLDNHFVNDPVTENPRLGSLEVWSIVNPNTRGTHPIHLHLVQFRVIDRRPFDTEV
YQSTGEIVYTGPNEAPPLHEQGYKDTIQAHAGEVIRIVARFVPTYTGRYVWHCHIL
EHEDYDMMRPMDIIQ (SEQ ID NO: 23)

[0196] In some embodiments, provided herein, engineered copper dehydrogenases are derived from BiLac: *Bacillus licheniformis* laccase (UniProtID: A0A6B9ET10). The wild-type *Bacillus licheniformis* laccase enzyme has an amino acid sequence set forth in SEQ ID NO:24. In some embodiments, the copper dehydrogenase comprises a sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 99.9% sequence identity to SEQ ID NO:24, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 103, 105, 151, 153, 421, 423, 490 and 492 of SEQ ID NO: 24 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 24.

[0197] Wild-type multicopper oxidase from BiLac: *Bacillus licheniformis* Laccase UniProtID: A0A6B9ET10

MKLEKFVDRLPIPVLPQSQSKEMTYEVTMKEFQQQLHRDLPPTRLFYNGV
YPGPTFEVQKHEKVAVRWLNKLPDRHFLPVDHTLHDDGHHEHEVKTVVHLHG
GCTPADSDGYPEAWYTKDFHAKGPFFEREVYEYPNEQDATAALWYHDHAMAIR
LNVYAGLVGLYFIRDREERSLNLPGGEYEIPLLIQDKSFHEDGSLFYPRQPDNPSP
DLPDPSIVPAFCGDTILVNGKVWPF AELEPRKYRFRILNASNTRIFELYFDHDITCH
QIGTDGGLLQHPVKVNELVIAPAERCDIIVDFSRAEGKTVTLKNRIGCGGQDADP
DTDADIMQFRISKPLKQKDTSSLPRILRKRPFYRRHKINALRNLSLGAAVDQYGR
PVLLLNNTKWHEPVTETPALGSTEIWSIINAGRAIHPIHLHLVQFMILDHRPFDIER
YQENGELVFTGPAVSPAPNEKGLKDTVKVPPGSVTRIIATFAPYSGRYVWHCHIL
EHEDYDMMRPLEVTDVRHQ (SEQ ID NO: 24)

[0198] In some embodiments, provided herein, engineered copper dehydrogenases are derived from ScLac: *Streptomyces cyaneus* laccase (UniProtID: F6L7B5). The wild-type *Streptomyces cyaneus* laccase enzyme has an amino acid sequence set forth in SEQ ID NO:25. In

some embodiments, the copper dehydrogenase comprises a sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 99.9% sequence identity to SEQ ID NO:25, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 145, 147, 185, 187, 511, 513, 586 and 588 of SEQ ID NO: 25 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 25.

[0199] *Wild-type multicopper oxidase from ScLac: Streptomyces cyaneus* Laccase
UniProtID: F6L7B5 (multi copper oxidase)

MTDIIERLTDS DGKPEEEQLGTGELTPYTAPLPVPPVLRPASDDVLHETEIALRPA
WVRLHPQLPPTLMWGYDGQVPGPTIEVRRGQRVRIAWTNRIPKDSEYPVTSVEV
PLRTDGRPQSTTEPGREGVEPNKDVAALPAWSVTHLHGAQTGGGNDGWADNA
VGFGDAQLSEYPNDHQA VQWWYHDHAMNITRWNVMTGLYGYTLVRDDEEDA
LHLPCGEREIPLLLADRNLDTDEDGRLNGRLLHKTLIVQQQNPETGKPVSI PFSGP
YNTVNGRIWPYADVDDAWYRFRLVNASNARIYDLVLVDEDDNPVPGIVHQIGS
DGGLLPRPVPVDFD GALPTLTAAPAERFDLLVDFRGLAGRRLRLVNKGRNQPPG
VSDPAGDVRYPAVMEFRVRESCETDTFELPEVLSGSFRRLTHDIEHGHRLIVLTPP
ATKGGGGHPEIWEMTEVQNP GDIQVPTEGVIQVTGADGKTKTYRRTARTFNDGL
GFTIAEGSHEQWSFLNLAPIVHPMHIHLADFQLLGRDAYDVSGFDPAIGGTRSPIR
HDAGTTIPLAPNELGHKDVFRVPGNQILRVMGKFDGAYGRFMYHCHLLEHEDM
GMMRPFVVMPEALKFDHGAGHGGHGDHGHGAGHTG (SEQ ID NO: 25)

[0200] In embodiments, provided herein, engineered copper dehydrogenases are derived from SiLac: *Streptomyces lavendulae* Laccase (UniProtID: Q8GB87). The wild-type enzyme has an amino acid sequence set forth in SEQ ID NO:26. In some embodiments, the copper dehydrogenase comprises a sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 99.9% sequence identity to SEQ ID NO:26, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 145, 147, 185, 187, 514, 516, 589 and 591 of SEQ ID NO: 26 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 26.

[0201] *Wild-type multicopper oxidase from SILac: Streptomyces lavendulae* Laccase
UniProtID: Q8GB87

MTDIIERITDGLAGAARDAAPAAADGELTPYAAPLTVPPVLRPDSTDVLRETEIAL
RPTWVRLHPQLPPTLMWGYGGSVPGPTVEVRRGQQRVRIAWTNRIPKGSEYPVTA
VEVPAANPNPSTEPGRGGVPPIADVAALPAWTVTHLHGAQTGGGNDGWADNA
VGYGDAQLSEYPNDHQAQVQWWYHDHAMNVTRWNVHTGLYGYTLVRDDEED
ALQLPSGKREIPLLIADRNLDTDDEGALNGRLLHKTIVVQEKNPETGKPVSVPF
GPYTTVNGRIWPYAEVDAAWHRFRLVNASNARIYDLVLIDEDDNPVPGVLHQIG
SDGGLLPRVPVDFDEALPTLTVAPAERMDLLIDFRALAGRRLRLVNKGAGQAP
GVPDPANNVRYPHVMEFRVGECGTDDPFELPEVLSGSFRRLTHDIEHGHRLIVLT
PPATKGGGGHPEIWEMTEVEDAEQVELPADGVIQLMSSEGALKTYRRTSRTFND
GLGFTVAEGSYEQWSFLNLAVNPPVVHPMHIHLADFQILGRDTYDVSGFDVTAG
GTRAPLAPDPATPVPLPPNERGYKDVFRALPGQMLRVMGRFDGAYGRFMYHCH
LLEHEDMGMMRPFVVMPEAMKFDHGAGHGGHGGHGGHGG (SEQ ID NO: 26)

II. Definitions

[0202] Unless otherwise defined, all terms (including technical and scientific terms) used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. It will be further understood that terms, such as those defined in commonly used dictionaries, should be interpreted as having a meaning that is consistent with their meaning in the context of the present application and relevant art and should not be interpreted in an idealized or overly formal sense unless expressly so defined herein. The terminology used in the description of the invention herein is for the purpose of describing particular aspects only and is not intended to be limiting of the invention. In case of a conflict in terminology, the present specification is controlling.

[0203] The term “subject” refers to a mammal (e.g., a human) in need of a L-DOPA concentration analysis. The subject may include dogs, cats, pigs, cows, sheep, goats, horses, rats, mice, non-human mammals, and humans. The term “subject” does not necessarily exclude an individual that is healthy in all respects and does not have or show signs of Parkinson’s Disease.

[0204] As used herein, the term “physiological conditions” refers to the range of conditions of temperature, pH, and tonicity (or osmolality) normally encountered within tissues in the body of a living human.

[0205] The term “*in vitro*” refers to artificial environments and to processes or reactions that occur within an artificial environment (e.g., a test tube).

[0206] The term “*in vivo*” refers to natural environments (e.g., a cell or organism or body) and to processes or reactions that occur within a natural environment.

[0207] As used herein, the term “and/or” refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative (“or”).

[0208] The term “or” refers to any one member of a particular list.

[0209] Unless otherwise apparent from the context, the term “about” encompasses values within a standard margin of error of measurement (e.g., SEM) of a stated value or variations $\pm$ 0.5%, 1%, 5%, or 10% from a specified value.

[0210] The singular forms of the articles “a,” “an,” and “the” include plural references unless the context clearly dictates otherwise. For example, the term “a protein” or “at least one protein” can include a plurality of proteins, including mixtures thereof.

[0211] Compositions or methods “comprising” or “including” one or more recited elements may include other elements not specifically recited. For example, a composition that “comprises” or “includes” a protein may contain the protein alone or in combination with other ingredients.

[0212] It is understood that where a parameter range is provided, all integers and ranges within that range, and tenths and hundredths thereof, are also provided by the embodiments. For example, “5-10%” includes 5%, 6%, 7%, 8%, 9%, and 10%; 5.0%, 5.1%, 5.2%....9.8%, 9.9%, and 10.0%; and 5.00%, 5.01%, 5.02%....9.98%, 9.99%, and 10.00%, as well as, for example, 6-9%, 8-10%, 5.1%-9.9%, and 5.01%-9.99%. Similarly, where a list is presented, unless stated otherwise, it is to be understood that each individual element of that list, and every combination of components of that list, is a separate embodiment. For example, “1, 2, 3, 4, and 5” encompasses, among numerous embodiments, 1; 2; 3; 1 and 2; 3 and 5; 1, 3, and 5; and 1, 2, 4, and 5. All ranges are inclusive of their endpoints unless otherwise stated.

[0213] Other definitions are provided below.

III. Compositions

Engineered Copper Dehydrogenases

[0214] In one embodiment, an isolated, engineered copper dehydrogenase that exhibits decreased oxidase activity towards oxygen while substantially retaining dehydrogenase (Dh) activity when compared to a wild-type multicopper oxidase (MCO) is provided. In embodiments, an engineered copper dehydrogenase exhibits increased catalytic activity compared to a wild-type MCO. In some embodiments, an engineered copper dehydrogenases further exhibits increased activity to dehydrogenate L-DOPA as a substrate.

[0215] As used herein, “isolated,” with respect to a polypeptide (and also a polynucleotide), means a molecule (e.g., polypeptide, protein or polynucleotide) isolated from its natural environment or prepared using synthetic methods such as those known to one of skill in the art. Complete purification is not required in either case. The molecules described herein can be isolated and purified from normally associated material in conventional ways, such that in the purified preparation the molecule is the predominant species in the preparation. At the very least, the degree of purification is such that extraneous material in the preparation does not interfere with use of the molecule in the manner disclosed herein. The molecule is at least about 85% pure; alternatively, at least about 90% pure, alternatively, at least about 95% pure; and alternatively, at least about 99% pure.

[0216] As used herein, “about” means within a statistically meaningful range of a value or values such as a stated concentration, length, molecular weight, pH, sequence identity, time frame, temperature or volume. Such a value or range can be within an order of magnitude, typically within 20%, more typically within 10%, and even more typically within 5% of a given value or range. The allowable variation encompassed by “about” will depend upon the particular system under study, and can be readily appreciated by one of skill in the art.

[0217] The term “wild-type” refers to entities having a structure and/or activity as found in a normal (as contrasted with mutant, diseased, altered, or so forth) state or context. Wild-type genes and polypeptides often exist in multiple different forms (e.g., alleles). A “wild-type amino acid residue” at a given position refers to the amino acid present at a given position in a wild-type polypeptide.

[0218] As used herein, “mutant,” when used in connection with a polypeptide or protein such as an enzyme, means a variant containing a substitution in one or more of the amino acid

residues on the polypeptide or protein at the indicated position(s) or a deletion of one or more amino acids at the indicated position(s). Mutant also is used for a polynucleotide encoding such a mutant polypeptide or protein.

[0219] As used herein, “a position corresponding to” means the position of an amino acid residue in a query amino acid sequence that is aligned with the amino acid residue in a reference amino acid sequence using software such as AlignX of Vector NTI with default parameters (available from Invitrogen; see, Lu & Moriyama (2004) *Brief Bioinform.* 5:378-88). Thus, “amino acid (AA) residue at a position corresponding to the position Y of the amino acid sequence set forth in SEQ ID NO: X” means the AA residue in a query amino acid sequence that is aligned with AA Y of SEQ ID NO: X when the query amino acid sequence is aligned with SEQ ID NO: X using AlignX of Vector NTI with default parameters. It should be noted that the AA Y of SEQ ID NO: X itself is also encompassed by this term.

[0220] As used herein, “oxidase activity” means an enzymatic activity of the engineered copper dehydrogenase to utilize oxygen as an electron acceptor. Engineered copper dehydrogenases with repressed oxidase activity have a reduced ability to transfer electrons to oxygen compared to wild-type MCOs which readily reduce oxygen to water. The oxidase activity may be assayed using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as a substrate by monitoring the increase in absorbance at 420nm based on the formation of reduced ABTS, and/or by any method known in the art.

[0221] As used herein, “dehydrogenase activity” or “Dh activity” means an enzymatic activity of the engineered copper dehydrogenase to catalyze the oxidation of L-DOPA to L-dopaquinone.

[0222] As used herein, “copper dehydrogenase” (CoDh) means an enzyme engineered from a multicopper oxidase (MCO) that exhibits reduced oxidase activity while substantially retaining dehydrogenase (Dh) activity. “Copper dehydrogenase” may be used interchangeably with “engineered MCO mutant” or “engineered PaMCO mutant.”

[0223] As used herein, “L-DOPA dehydrogenase” (L-DOPADh) means a copper dehydrogenase or engineered MCO mutant capable of oxidizing L-DOPA as a substrate. A L-DOPADh may exhibit high substrate specificity towards L-DOPA. “L-DOPA dehydrogenase” may be used interchangeably with “copper dehydrogenase.”

[0224] As used herein, “biosensor” means a device containing bio-recognition elements (e.g., enzymes, aptamers, antibodies, etc.) that react with a target molecule and then transduce the biological signal into an electrical signal that can be analyzed.

[0225] It is therefore desired with respect to electrochemical biosensors to modulate the engineered enzyme’s activity towards L-DOPA and away from oxygen as an electron acceptor. In one embodiment, the copper dehydrogenase therefore has a reduced oxidase activity towards oxygen when compared to a wild-type MCO, while retaining the dehydrogenase activity towards L-DOPA. In another embodiment, the engineered copper dehydrogenase can have an oxidase activity towards oxygen of about 99.9% or less when compared to the oxidase activity wild-type MCO. In another embodiment, the oxidase activity of the engineered copper dehydrogenase is reduced by at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% as compared to the wild-type multicopper oxidase. In embodiments, engineered MCOs can directly transfer electrons to an electrode. In addition, the engineered copper dehydrogenase can have a dehydrogenase activity increased by about 50% or more when compared to a wild-type MCO when tested in a solution containing about 20-400 μ M L-DOPA in the presence of ambient oxygen (*see* Figure 5 comparing the current generated by the mutant PaMCO enzymes based on L-DOPA concentration). Alternatively, dehydrogenase activity of the copper dehydrogenase is increased by at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 100% as compared to the dehydrogenase activity of the wild-type multicopper oxidase.

[0226] It should be understood that the numbering of the position of the amino acid sequence for engineered copper dehydrogenases herein begins at an initial Met and that the claimed copper dehydrogenases may or may not have the initial signal peptide sequence. Examples of amino acid sequences for the engineered MCOs include, those having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% sequence identity to any one of SEQ ID NOs: 1-6.

[0227] In embodiments, provided herein is an engineered copper dehydrogenase having at least 90% sequence identity to any one of SEQ ID NOs: 1-6, provided at least one of positions 64, 66, 104, 106, 366, 368, 431, and 433 is different from the histidine occupying the corresponding

position in SEQ ID NO:7. In embodiments, provided herein is an engineered copper dehydrogenase having at least 90% sequence identity to SEQ ID NO:7, provided at least one of positions 64, 66, 104, 106, 366, 368, 431, and 433 are different from the histidine residues in the corresponding position in SEQ ID NO:7.

[0228] In embodiments, an engineered copper dehydrogenase is provided, comprising a modification at i) a position corresponding to position 66 of the amino acid sequence set forth in SEQ ID NO: 7, wherein the modification includes a substitution of the wild-type histidine with a different amino acid residue; ii) a position corresponding to position 104 of the amino acid sequence set forth in SEQ ID NO: 7, wherein the modification includes a substitution of the wild-type histidine with a different amino acid residue; iii) a position corresponding to position 368 of the amino acid sequence set forth in SEQ ID NO: 7, wherein the modification includes a substitution of the wild-type histidine with a different amino acid residue; or iv) a position corresponding to position 431 of the amino acid sequence set forth in SEQ ID NO: 7, wherein the modification includes a substitution of the wild-type histidine with a different amino acid residue.

[0229] In embodiments, an engineered copper dehydrogenase is provided, comprising a modification at i) a position corresponding to position 368 of the amino acid sequence set forth in SEQ ID NO: 7, wherein the modification includes a substitution of the wild-type histidine with a different amino acid residue and ii) a position corresponding to position 431 of the amino acid sequence set forth in SEQ ID NO: 7, wherein the modification includes a substitution of the wild-type histidine with a different amino acid residue.

[0230] In embodiments, an engineered copper dehydrogenase is provided, comprising a modification at i) a position corresponding to position 66 of the amino acid sequence set forth in SEQ ID NO: 7, wherein the modification includes a substitution of the wild-type histidine with a different amino acid residue; ii) a position corresponding to position 368 of the amino acid sequence set forth in SEQ ID NO: 7, wherein the modification includes a substitution of the wild-type histidine with a different amino acid residue; and iii) a position corresponding to position 431 of the amino acid sequence set forth in SEQ ID NO: 7, wherein the modification includes a substitution of the wild-type histidine with a different amino acid residue.

[0231] In embodiments, an engineered copper dehydrogenase is provided, comprising a modification at i) a position corresponding to position 66 of the amino acid sequence set forth in SEQ ID NO: 7, wherein the modification includes a substitution of the wild-type histidine with an

alanine amino acid residue; ii) a position corresponding to position 104 of the amino acid sequence set forth in SEQ ID NO: 7, wherein the modification includes a substitution of the wild-type histidine with an alanine amino acid residue; iii) a position corresponding to position 368 of the amino acid sequence set forth in SEQ ID NO: 7, wherein the modification includes a substitution of the wild-type histidine with an alanine amino acid residue; or iv) a position corresponding to position 431 of the amino acid sequence set forth in SEQ ID NO: 7, wherein the modification includes a substitution of the wild-type histidine with an alanine amino acid residue.

[0232] In embodiments, an engineered copper dehydrogenase is provided, comprising a modification at i) a position corresponding to position 368 of the amino acid sequence set forth in SEQ ID NO: 7, wherein the modification includes a substitution of the wild-type histidine with an alanine amino acid residue and ii) a position corresponding to position 431 of the amino acid sequence set forth in SEQ ID NO: 7, wherein the modification includes a substitution of the wild-type histidine with an alanine amino acid residue.

[0233] In embodiments, an engineered copper dehydrogenase is provided, comprising a modification at i) a position corresponding to position 66 of the amino acid sequence set forth in SEQ ID NO: 7, wherein the modification includes a substitution of the wild-type histidine with an amino acid alanine residue; ii) a position corresponding to position 368 of the amino acid sequence set forth in SEQ ID NO: 7, wherein the modification includes a substitution of the wild-type histidine with an alanine amino acid residue; and iii) a position corresponding to position 431 of the amino acid sequence set forth in SEQ ID NO: 7, wherein the modification includes a substitution of the wild-type histidine with an alanine amino acid residue.

[0234] “Sequence identity” or “identity” in the context of two polynucleotides or polypeptide sequences refers to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have “sequence similarity” or “similarity.” Means for making

this adjustment are well known to those of skill in the art. Typically, this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

[0235] “Percentage of sequence identity” refers to the value determined by comparing two optimally aligned sequences (greatest number of perfectly matched residues) over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity. Unless otherwise specified (e.g., the shorter sequence includes a linked heterologous sequence), the comparison window is the full length of the shorter of the two sequences being compared.

[0236] Unless otherwise stated, sequence identity/similarity values refer to the value obtained using GAP Version 10 using the following parameters: % identity and % similarity for a nucleotide sequence using GAP Weight of 50 and Length Weight of 3, and the nwsgapdna.cmp scoring matrix; % identity and % similarity for an amino acid sequence using GAP Weight of 8 and Length Weight of 2, and the BLOSUM62 scoring matrix; or any equivalent program thereof. “Equivalent program” includes any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by GAP Version 10.

[0237] The term “conservative amino acid substitution” refers to the substitution of an amino acid that is normally present in the sequence with a different amino acid of similar size, charge, or polarity. Examples of conservative substitutions include the substitution of a non-polar (hydrophobic) residue such as isoleucine, valine, or leucine for another non-polar residue. Likewise, examples of conservative substitutions include the substitution of one polar

(hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, or between glycine and serine. Additionally, the substitution of a basic residue such as lysine, arginine, or histidine for another, or the substitution of one acidic residue such as aspartic acid or glutamic acid for another acidic residue are additional examples of conservative substitutions. Examples of nonconservative substitutions include the substitution of a non-polar (hydrophobic) amino acid residue such as isoleucine, valine, leucine, alanine, or methionine for a polar (hydrophilic) residue such as cysteine, glutamine, glutamic acid or lysine and/or a polar residue for a non-polar residue. Typical amino acid categorizations are summarized below.

Alanine	Ala	A	Nonpolar	Neutral	1.8
Arginine	Arg	R	Polar	Positive	-4.5
Asparagine	Asn	N	Polar	Neutral	-3.5
Aspartic acid	Asp	D	Polar	Negative	-3.5
Cysteine	Cys	C	Nonpolar	Neutral	2.5
Glutamic acid	Glu	E	Polar	Negative	-3.5
Glutamine	Gln	Q	Polar	Neutral	-3.5
Glycine	Gly	G	Nonpolar	Neutral	-0.4
Histidine	His	H	Polar	Positive	-3.2
Isoleucine	Ile	I	Nonpolar	Neutral	4.5
Leucine	Leu	L	Nonpolar	Neutral	3.8
Lysine	Lys	K	Polar	Positive	-3.9
Methionine	Met	M	Nonpolar	Neutral	1.9
Phenylalanine	Phe	F	Nonpolar	Neutral	2.8
Proline	Pro	P	Nonpolar	Neutral	-1.6
Serine	Ser	S	Polar	Neutral	-0.8
Threonine	Thr	T	Polar	Neutral	-0.7
Tryptophan	Trp	W	Nonpolar	Neutral	-0.9
Tyrosine	Tyr	Y	Polar	Neutral	-1.3
Valine	Val	V	Nonpolar	Neutral	4.2

Engineered Copper Dehydrogenase-Encoding Polynucleotides

[0238] In one embodiment, an isolated polynucleotide that encodes for an engineered copper dehydrogenase is described herein.

[0239] The terms “nucleic acid” and “polynucleotide,” used interchangeably herein, refer to polymeric forms of nucleotides of any length, including ribonucleotides, deoxyribonucleotides, or analogs or modified versions thereof. They include single-, double-, and multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, and polymers comprising purine bases,

pyrimidine bases, or other natural, chemically modified, biochemically modified, non-natural, or derivatized nucleotide bases.

[0240] Nucleic acids are said to have “5’ ends” and “3’ ends” because mononucleotides are reacted to make oligonucleotides in a manner such that the 5’ phosphate of one mononucleotide pentose ring is attached to the 3’ oxygen of its neighbor in one direction via a phosphodiester linkage. An end of an oligonucleotide is referred to as the “5’ end” if its 5’ phosphate is not linked to the 3’ oxygen of a mononucleotide pentose ring. An end of an oligonucleotide is referred to as the “3’ end” if its 3’ oxygen is not linked to a 5’ phosphate of another mononucleotide pentose ring. A nucleic acid sequence, even if internal to a larger oligonucleotide, also may be said to have 5’ and 3’ ends. In either a linear or circular DNA molecule, discrete elements are referred to as being “upstream” or 5’ of the “downstream” or 3’ elements.

[0241] The polynucleotide encoding the wild-type MCO may be cloned from the genome of respective organisms using PCR or other known techniques. Then, mutations may be introduced by techniques such as site-directed mutagenesis, PCR mutagenesis or any other known techniques. The amino acid residue to be mutated may be identified using any software for sequence alignment available in the art. Alternatively, polynucleotides coding for the engineered copper dehydrogenase may be prepared by PCR using a series of chemically synthesized oligonucleotides, or fully synthesized. Examples of nucleotide sequences for the engineered copper dehydrogenase can include, but are not limited to, those encoding an amino acid sequence as set forth in any one of SEQ ID NOs: 1-6 modified at least at one of a position corresponding to position 66, 104, 368, and 431 of SEQ ID NO: 7.

[0242] In some embodiments, the wild-type *Pyrobaculum aerophilum* multicopper oxidase is encoded by the DNA sequence provided in SEQ ID NO: 9.

[0243] *Wild-type multicopper oxidase from Pyrobaculum aerophilum*

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atgactggtgaagtcaagaggcctgagacatccgcgctgtcccccgttaattaaagaggcgacatatattgaggccactgcc
agtggatatatggcagaggaggtattaaatcccacaataatacttaggcgtggacaaagagttgatatgacattaaaaataaact
cactgagccgaccattgtccactggcacggctttgatgtaaaactggcataacgatgcgcatccctccttcgccataacgccgggg
gagagctacaattattcttcgacgttgtgaatagggtggcacatatctctatccccccaccccatgggctgaccgcaaaaca
gttctacatgggacaactaggtctcgtcatagtgaggactcgggtctgatctcgggtttaatacggcgtaaacgatctgccgc
tggtgatatctgacaggagatttataggcggagcgcccggttacaacccgacgccaatggagatgatcgctgggttttgggaaa
```

cgctgttttagtaaaccggcggttaaagacgccgtatttaaactatcaggagggagctacagactcaggctcgtaaattggctctaacg
 cgaggctgtatatgctctctattgttaagaaaaacggcgatgtgtgcccatgaggcttattgccgttgaccagggcttttggccag
 gccaatagaggtaaggcggttattcctcgcgccagctgagagggctgagggttggttagagctgggcgagggcggttacctctt
 gaagaatacgccttttgatcccatgcatttagaaatgggccacgggatgcaagaggcgctccccgagggctctgagtacacaat
 agccactttcttagttgaaggcaaggcgaggcggtgcctgtggaggccttgtctgatccgccccagagccccaaaaacca
 ctgcacagaggagggttgcgttatctctatcaggaatgcagtggacgataaacggcatgttctggaatgcctcaaaccgctgttt
 gaacacgtatctgtagagggcgctgagctgtgggaaatagtaacgacaaggcgtctatgccccaccctatgcacttacacggc
 ttcccatgtggataattgagcgtaaagacagcccaagacaagtggcggaacttgccgtggacaataggggcagactccccac
 agatctcggctgaaagacacagtgttaatatggccaggcgagacagttaaaatcgttgtgaattttgacgaaaaagaggggg
 caactctccattccactgccacaatttagaacaatgaagacggggcgatgatataacatagcagttaaataa (SEQ ID
 NO: 9)

[0244] An embodiment is a *Pyrobaculum aerophilum* multicopper oxidase comprising a Phe290Ile mutation to improve catalytic activity, which corresponds to a mutation within the codon at positions 784-786 of the DNA sequence set forth in SEQ ID NO: 10.

[0245] *Multicopper oxidase from Pyrobaculum aerophilum with Phe290Ile*

atgactggtgaagtcaagaggcctgagacatccgcgctgtcccccgtaattaaagaggcgacatatattgaggccactgcc
 agtggatatattggcagaggagattataatcccacaataatacttaggcgtggacaaagagttgatatgacattaaaaataaact
 cactgagccgaccattgtccactggcacggctttgatgtaaactggcataacgatgcgcacccctcctcgccataacgccgggg
 gagagctacaattattcttgcagttgtgaatagggtggcacatatctctatcacccccacccccatgggctgaccgcaaaaca
 gttctacatgggacaactaggtctcgtcatagtggaggactcggggctctgatctcgggtttaaatacggcgtaaacgatctgccgc
 tgggtatatctgacaggagattataggcggagcggcgtttacaacccgacgccaatggagatgatcgtgggttttgggaaa
 cgctgttttagtaaaccggcggttaaagacgccgtatttaaactatcaggagggagctacagactcaggctcgtaaattggctctaacg
 cgaggctgtatatgctctctattgttaagaaaaacggcgatgtgtgcccatgaggcttattgccgttgaccagggcttttggccag
 gccaatagaggtaaggcggttattcctcgcgccagctgagagggctgagggttggttagagctgggcgagggcggttacctctt
 gaagaatacgcctattgatcccatgcatttagaaatgggccacgggatgcaagaggcgctccccgagggctctgagtacacaat
 agccactttcttagttgaaggcaaggcgaggcggtgcctgtggaggccttgtctgatccgccccagagccccaaaaacca
 ctgcacagaggagggttgcgttatctctatcaggaatgcagtggacgataaacggcatgttctggaatgcctcaaaccgctgttt
 gaacacgtatctgtagagggcgctgagctgtgggaaatagtaacgacaaggcgtctatgccccaccctatgcacttacacggc
 ttcccatgtggataattgagcgtaaagacagcccaagacaagtggcggaacttgccgtggacaataggggcagactccccac
 agatctcggctgaaagacacagtgttaatatggccaggcgagacagttaaaatcgttgtgaattttgacgaaaaagaggggg

caactcttccattcc**act**gccacaatttagaacaatgaagacgggggcatgatgataaacatagcagttaaataa (SEQ ID NO: 10)

[0246] In one embodiment, the engineered copper dehydrogenase derived from *Pyrobaculum aerophilum* harboring a Phe290Ile mutation to increase catalytic activity and a His94Ala mutation to reduce oxidase activity towards oxygen is encoded by the DNA sequence set forth in SEQ ID NO: 11. The Phe290Ile mutation corresponds to a mutation within the codon at positions 784-786 and the His94Ala mutation corresponds to a mutation within the codon at positions 196-198 of the DNA sequence set forth in SEQ ID NO: 11.

[0247] *Copper dehydrogenase derived from Pyrobaculum aerophilum with His94Ala/Phe290Ile*

atgactggtgaagtcaagaggcctgagacatccgcgctgtcccccgttaattaaagaggcgacatatattgaggccactgcc
agtgatataatggcagaggagattaaatcccacaataatacttaggcgtggacaaagagttgatatgacattaaaaataaact
cactgagccgaccattgtccactgggcccgtttgatgtaaactggcataacgatgcgcacccctcttcgccataacgccggg
gagagctacaattattcttcgacgttgtgaatagggtggcacatatctctatcacccccacccccatgggctgaccgcaaaaca
gttctacatgggacaactaggtctcgtcatagtgaggactcggggctctgatctcgggtttaaatacggcgtaaacgatctgccgc
tggtgatatactgacaggagattataggcggagcggcgtttacaacccgacgccaatggagatgatcgctgggtttttgggaaa
cgctgttttagtaaaggcggttaaagacggcgtatttaaactatcaggaggagctacagactcaggctcgtaaatggctctaagc
cgaggctgtatatgctctctattgtaagaaaaacggcgatgttgtgccatgaggctattgccgttgaccagggtttttggcag
gccaatagaggttaaggcggttattcctcgcgccagctgagagggtgaggttggttagagctgggcgaggcggtttacctctt
gaagaatacgcctattgatcccatgcatttagaaatgggccacgggatgcaagaggcgctccccgagggtctctgagtacacaat
agccactttcttagttgaaggcaaggcgaggcgggtgctgtggaggccttctgatccgccccagagccccaaaaccca
ctcgcacgaggaggtttgcgttatctctatcaggaatgcagtggacgataaacggcatgttctggaatgcctcaaaccgcgtgtt
gaacacgtatctgtagaggcgctcagctgtgggaaatagttaacgacaaggcgtctatgccccaccctatgcactt**ac**ggc
ttcccatgttgataattgagcgtaaagacagcccaagacaagtggcggaaacttgccgtggacaataggggcagactccccac
agatctcggcttgaaagacacagtgttaatatggccaggcgagacagttaaatcgttgtgaattttgacgaaaaagaggggg
caactcttccattcc**act**gccacaatttagaacaatgaagacgggggcatgatgataaacatagcagttaaataa (SEQ ID NO: 11)

[0248] In another embodiment, the engineered copper dehydrogenase derived from *Pyrobaculum aerophilum* harboring a Phe290Ile mutation to increase catalytic activity and a His132Ala mutation to reduce oxidase activity towards oxygen is encoded by the DNA sequence set forth in SEQ ID NO: 12. The Phe290Ile mutation corresponds to a mutation within the codon

at positions 784-786 and the His132Ala mutation corresponds to a mutation within the codon at positions 310-312 of the DNA sequence set forth in SEQ ID NO: 12.

[0249] *Copper dehydrogenase derived from Pyrobaculum aerophilum with His132Ala/Phe290Ile*

atgactggtgaagtcaagaggcctgagacatccgcgctgtcccccgtaattaaagaggcgacatatattgaggccactgcc
 agtggatatatggcagaggagattataatcccacaataatacttaggcgtggacaaagagttgatatgacattaaaaataaact
 cactgagccgaccattgtccactggcacggctttgatgtaactggcataacgatgcgcacccctcctcgccataacgccgggg
 gagagctacaattattcttcgacgttgaatagggtggcacatatctctatgccccacccccatgggctgaccgcaaaaca
 gttctacatgggacaactaggtctcgtcatagtggaggactcggggctgatctcgggtttaatacggcgtaaacgatctgccgc
 tgggtatatctgacaggagattataggcggagcggcgtttacaacccgacgccaatggagatgatcgctgggttttgggaaa
 cgctgttttagtaaaggcggttaaagacggcgtatttaaactatcaggaggagctacagactcaggctcgtaaatggctctaacg
 cgaggctgtatatgctctctattgtaagaaaaacggcgatgttgccccatgaggctattgccgttgaccagggttttggccag
 gccaatagaggaaggcggtattcctcgcgccagctgagagggtgaggttggttagagctggcgaggcggtttacctctt
 gaagaatacgcctattgatcccatgcatttagaaatgggccacgggatgcaagaggcgctccccgagggtctgagtacacaat
 agccactttcttagttgaaggcaaggcgaggcgggtgcctgtggaggccttgtctgatccgccccagagccccaaaaccca
 ctgcacgaggaggtttgcttatctctatcaggaatgcagtggacgataaacggcatgttctggaatgcctcaaacccgctgttt
 gaacacgtatctgtagaggcgctgagctgtgggaaatagttaacgacaaggcgtctatgccccaccctatgcacttaacggc
 ttcccatgtggataattgagcgtaaagacagcccaagacaagtggcggaaactgccgtggacaataggggcagactccccac
 agatctcggtgaaagacacagtgttaatatggccaggcgagacagttaaatcgttgtaattttgacgaaaaagaggggg
 caactcttccattccactgccacaattagaacatgaagacgggggcatgatgataacatagcagttaaataa (SEQ ID
 NO: 12)

[0250] In one embodiment, the engineered copper dehydrogenase derived from *Pyrobaculum aerophilum* harboring a Phe290Ile mutation to increase catalytic activity and a His396Ala mutation to reduce oxidase activity towards oxygen is encoded by the DNA sequence set forth in SEQ ID NO: 13. The Phe290Ile mutation corresponds to a mutation within the codon at positions 784-786 and the His396Ala mutation corresponds to a mutation within the codon at positions 1102-1104 of the DNA sequence set forth in SEQ ID NO: 13.

[0251] *Copper dehydrogenase derived from Pyrobaculum aerophilum with Phe290Ile/His396Ala*

atgactggtgaagtcaagaggcctgagacatccgcgctgtcccccgtaattaaagaggcgacatatattgaggccactgcc
 agtggatatatggcagaggagattataatcccacaataatacttaggcgtggacaaagagttgatatgacattaaaaataaact

cactgagccgaccattgtccactggcacggctttgatgtaaactggcataacgatgcgcacccctcctcgccataacgccgggg
gagagctacaattattctttcgacgttgaatagggtggcacatatctctatcaccaccaccccatgggctgaccgcaaaaca
gttctacatgggacaactaggtctcgtcatagtggaggactcggggctgatctcgggtttaaatacggcgtaaaccgatctgccgc
tggtgatatctgacaggagattataggcggagcgccggttacaacccgacgccaatggagatgatcgctgggttttgggaaa
cgctgttttagtaaaccggcgtaaagacgccgtatttaaactatcaggaggagctacagactcaggctcgtaaatggctctaacg
cgaggctgtatatgctctctattgttaagaaaaacggcgatgtgtgccatgaggcttattgccgttgaccagggttttggccag
gccaatagaggtaaggcggtattcctcgccagctgagagggtgagggttggttagagctggcgaggcggtttacctctt
gaagaatacgccattgatcccatgcatttagaaatgggccacgggatgcaagaggcgctccccgagggtctgagtacacaat
agccactttcttagttgaaggcaaggcgaggcggtgcctgtggaggccttctgatccgccccagagccccaaaaccca
ctcgcacgaggaggttgcgttatctctatcaggaatgcagtggacgataaacggcatgttctggaatgcctcaaacccgctgttt
gaacacgtatctgtagaggcgctgagctgtgggaaatagttaacgacaaggcgtctatgccccaccctatgcacttagccggc
tttcccatgtggataattgagcgtaaagacagcccaagacaagtggcggaacttgccgtggacaataggggcagactccccac
agatctcggttgaaagacacagtgttaatatggccaggcgagacagttaaatcgttgaattttgacgaaaaagaggggg
caactcttccattccactgccacaattagaacatgaagacgggggcatgatgataaacatagcagttaaataa (SEQ ID
NO: 13)

[0252] In another embodiment, the engineered copper dehydrogenase derived from *Pyrobaculum aerophilum* harboring a Phe290Ile mutation to increase catalytic activity and a His459Ala mutation to reduce oxidase activity towards oxygen is encoded by the DNA sequence set forth in SEQ ID NO: 14. The Phe290Ile mutation corresponds to a mutation within the codon at positions 784-786 and the His459Ala mutation corresponds to a mutation within the codon at positions 1291-1293 of the DNA sequence set forth in SEQ ID NO: 14.

[0253] *Copper dehydrogenase derived from Pyrobaculum aerophilum with Phe290Ile/His459Ala*

atgactggtgaagtcaagaggcctgagacatccgcgctgtcccccgtaattaaagaggcgacatatattgaggccactgcc
agtggatatatggcagaggaggtattaaatcccacaataatacttaggcgtggacaaagagttgatatgacattaaaaataaact
cactgagccgaccattgtccactggcacggctttgatgtaaactggcataacgatgcgcacccctcctcgccataacgccgggg
gagagctacaattattctttcgacgttgaatagggtggcacatatctctatcaccaccaccccatgggctgaccgcaaaaca
gttctacatgggacaactaggtctcgtcatagtggaggactcggggctgatctcgggtttaaatacggcgtaaaccgatctgccgc
tggtgatatctgacaggagattataggcggagcgccggttacaacccgacgccaatggagatgatcgctgggttttgggaaa
cgctgttttagtaaaccggcgtaaagacgccgtatttaaactatcaggaggagctacagactcaggctcgtaaatggctctaacg
cgaggctgtatatgctctctattgttaagaaaaacggcgatgtgtgccatgaggcttattgccgttgaccagggttttggccag

gccaatagaggtaagggcggtattcctcgccagctgagagggctgaggttggttagagctgggcgagggcggttacctctt
gaagaatacgcctattgatcccatgcatttagaaatgggccacgggatgcaagaggcgctccccgagggctctgagtacacaat
agccactttcttagttgaaggcaagggcgagggcggtgcctgtggaggccttgtctgatccgccccagagccccaaaaccca
ctcgcacgaggagggttgcgttatctctatcaggaatgcagtggacgataaacggcatgttctggaatgcctcaaacccgctgttt
gaacacgtatctgtagagggcgctgagctgtgggaaatagttaacgacaaggcgtctatgccccaccctatgcactta**acggc**
tttcccatgtggataattgagcgtaaagacagcccaagacaagtggcggaaacttgcctggacaataggggcagactccccac
agatctcggttgaaagacacagtgttaatatggccaggcgagacagttaaaatcgttgtaattttgacgaaaaagaggggg
caactcttccattc**gcct**gccacaatttagaacatgaagacgggggcatgatgataaacatagcagttaaataa (SEQ ID
NO: 14)

[0254] In one embodiment, the engineered copper dehydrogenase derived from *Pyrobaculum aerophilum* harboring a Phe290Ile mutation to increase catalytic activity and His396Ala/His459Ala mutations to reduce oxidase activity towards oxygen is encoded by the DNA sequence set forth in SEQ ID NO: 15. The Phe290Ile mutation corresponds to a mutation within the codon at positions 784-786, the His396Ala corresponds to a mutation within the codon at positions 1102-1104, and the His459Ala mutation corresponds to a mutation within the codon at positions 1291-1293 of the DNA sequence set forth in SEQ ID NO: 15.

[0255] *Copper dehydrogenase derived from Pyrobaculum aerophilum with Phe290Ile/His396Ala/His459Ala*

atgactggtgaagtcaagaggcctgagacatccgcgctgtcccccgtaattaaagaggcgacatatattgaggccactgcc
agtggatatatggcagaggagattaaatcccacaataatacttaggcgtggacaaagagttgatatgacattaaaaataaact
cactgagccgaccattgtccactggcacggctttgatgtaaactggcataacgatgcgcacccctcctcgccataacgccgggg
gagagctacaattattctttcgacgttgtaataagggtggcacatatctatcacccccacccccatgggctgaccgcaaaaca
gttctacatgggacaactaggtctcgtcatagtggaggactcggggctctgatctcgggtttaatacggcgtaaacgatctgccgc
tggtgatatctgacaggagatttataggcggagcggcgtttacaacccgacgccaatggagatgatcgctgggttttgggaaa
cgctgttttagtaaacggcggttaaagacggcgtatttaactatcaggaggagctacagactcaggctcgtaatggctctaacg
cgaggctgtatatgctctctattgttaagaaaaacggcgatgtgtgcccatgaggctattgccgttgaccagggccttttggccag
gccaatagaggtaagggcggtattcctcgccagctgagagggctgaggttggttagagctgggcgagggcggttacctctt
gaagaatacgcctattgatcccatgcatttagaaatgggccacgggatgcaagaggcgctccccgagggctctgagtacacaat
agccactttcttagttgaaggcaagggcgagggcggtgcctgtggaggccttgtctgatccgccccagagccccaaaaccca
ctcgcacgaggagggttgcgttatctctatcaggaatgcagtggacgataaacggcatgttctggaatgcctcaaacccgctgttt
gaacacgtatctgtagagggcgctgagctgtgggaaatagttaacgacaaggcgtctatgccccaccctatgcacttag**acggc**

tttcccatgttgataattgagcgtaaagacagcccaagacaagtggcggaacttgccgtggacaataggggcagactccccac
 agatctcggcttgaaagacacagtgttaatatggccaggcgagacagttaaaatcggtgtgaattttgacgcaaaaaagaggggg
 caactcttccattc**g**ctgccacaatttagaacatgaagacgggggcatgatgataaacatagcagttaaataa (SEQ ID
 NO: 15)

[0256] In another embodiment, the engineered copper dehydrogenase derived from *Pyrobaculum aerophilum* harboring a Phe290Ile mutation to increase catalytic activity and H94A/His396Ala/His459Ala mutations to reduce oxidase activity towards oxygen is encoded by the DNA sequence set forth in SEQ ID NO: 16. The Phe290Ile mutation corresponds to a mutation within the codon at positions 784-786, the His94Ala mutation corresponds to a mutation within the codon at positions 196-198, the His396Ala mutation corresponds to a mutation within the codon at positions 1102-1104, and the His459Ala mutation corresponds to a mutation within the codon at positions 1291-1293 of the DNA sequence set forth in SEQ ID NO: 16.

[0257] *Copper dehydrogenase derived from Pyrobaculum aerophilum with His94Ala/Phe290Ile/His396/His459Ala*

atgactggtgaagtcaagaggcctgagacatccgcgctgtcccccgtaattaaagaggcgacatatattgaggccactgcc
 agtggatatatggcagagggagtattaaatccacaataatacttaggcgtggacaaagagttgatatgacattaaaaataaact
 cactgagccgaccattgtccactgggcccggctttgatgtaaactggcataacgatgcgcacccctcctcgccataacgccgggg
 gagagctacaattattctttcgacgttgatgaatagggtggcacatatctatcacccccaccccatgggctgaccgcaaaaca
 gttctacatgggacaactaggtctcgtcatagtggaggactcggggtctgatctcgggtttaatacggcgtaaacgatctgccgc
 tgggtgatatctgacaggagattataggcggagcgcgggtttacaacccgacgccaatggagatgatcgctgggtttttgggaaa
 cgctgttttagtaaacggcgtaaaagacgccgtatttaaatatcaggaggagctacagactcaggctcgtaaatggctctaacg
 cgaggctgtatatgctctctattgttaagaaaaacggcgatgtgtgcccatgaggcttattgccgtgaccagggtttttggccag
 gccaatagaggtaagggcggtattcctcgcgccagctgagagggctgagggttggttagagctgggcgagggcggtttaccttt
 gaagaatacgcctattgatcccatgcatttagaaatgggccacgggatgcaagaggcgctccccgagggtctgagtacacaat
 agccactttcttagttgaaggcaagggcgaggcggtgcctgtggaggccttgtctgatccgccccagagccccaaaaacca
 ctcgcacgaggaggtttgcgttatctctatcaggaatgcagtggacgataaacggcatgttctggaatgcctcaaacccgctgttt
 gaacacgtatctgtagaggcgctcagctgtgggaaatagtaacgacaaggcgtctatgccccaccctatgcacttag**g**ccggc
 tttcccatgttgataattgagcgtaaagacagcccaagacaagtggcggaacttgccgtggacaataggggcagactccccac
 agatctcggcttgaaagacacagtgttaatatggccaggcgagacagttaaaatcggtgtgaattttgacgcaaaaaagaggggg
 caactcttccattc**g**ctgccacaatttagaacatgaagacgggggcatgatgataaacatagcagttaaataa (SEQ ID
 NO: 16)

Vectors and Host Cells

[0258] In another embodiment, provided herein is a vector comprising the engineered copper dehydrogenase-encoding polynucleotide or a host cell expressing the vector comprising the engineered copper dehydrogenase-encoding polynucleotide. Engineered copper dehydrogenases may be prepared by inserting an engineered or mutant polynucleotide into an appropriate expression vector and introducing the vector into an appropriate host cell, such as, for example, *Escherichia coli*. The transformant is cultured and the engineered copper dehydrogenase expressed in the transformant may be collected from the cells or culture medium by any known technique.

[0259] In some embodiments the expression vector is a pET-11a vector (SEQ ID NO: 17).

[0260] *pET-11a Expression Vector*

tttcatgtttgacagcttatcatcgataagctttaatgcggtagttatcacagttaaattgctaacgcagtcaggcaccgtgtatgaa
atctaaacaatgcgtcatcgtcatcctcggcaccgtcacctggatgctgtaggcataggcttggttatgccgtactgccgggcc
tcttgccgggatccggatatagttcctcctttcagcaaaaaacccctcaagaccggttagagggcccaaggggttatgctagtta
ttgctcagcgggtggcagcagccaactcagcttccttcgggctttagtagcagccgatccgcgacccatttctgtccaccagtc
atgctagccatatgtatatctccttctaaagttaaacaataattatttctagaggggaattgttatccgctcacaattcccctatagtga
gtcgtattaatttcgcgggatcgagatctcgatcctctacgccggacgcacgtggccggcatcaccggcgccacaggtgcgggt
tgctggcgcttatatcgccgacatcaccgatggggaagatcgggctcgccacttcgggctcatgagcgcttcttcggcggtggg
tatggtggcaggcccggtggccggggactgttggcgccatctccttgcatgcaccattccttcggcgggcggtgctcaacg
gctcaacctactactgggctgcttctaatagcaggagtcgcataagggagagcgtcgagatcccgacaccatcgatggcg
caaacctttcgcggtatggcatgatacgcccggaagagagtcattcagggtggtgaatgtgaaaccagtaacgttatagat
gtcgagagtatccgggtgtctcttatcagaccgtttcccgctgggtgaaccaggccagccacgtttctgcgaaaacgcgggaa
aaagtggaagcggcgatggcggagctgaattacattccaaccgcgtggcacaacaactggcgggcaaacagtcgttctgat
tgcggttgccacctcagcttgccctgcacgcgccgtcgcaaattgtcgcgcgattaaatctcgcgccgatcaactgggtgc
cagcgtggtggtgtcgatggtagaacgaagcggcgctgaagcctgtaaagcggcggtgcacaatcttctcgcgcaacgcgtca
gtgggctgatcattaactatccgctggatgaccaggatgccattgctgtggaagctgcctgactaatgttcggcggttatttctga
tgtcttgaccagaccccatcaacagtatttttctccatgaagacgggtacgcgactggcggtggagcatctggtcgcatggg
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attaacctataaaaataggcgatcacgaggcccttctgttcaagaa (SEQ ID NO: 17)

[0261] In embodiments, the engineered copper dehydrogenase thus obtained may be purified by any of the known purification techniques including, but not limited to, ion exchange column chromatography, affinity chromatography, liquid chromatography, filtration, ultrafiltration, salt precipitation, solvent precipitation, immunoprecipitation, gel electrophoresis, isoelectric electrophoresis and dialysis.

[0262] In embodiments, provided herein are isolated or purified polypeptides, proteins and polynucleotides for an engineered copper dehydrogenase, a vector comprising the polynucleotide encoding the engineered copper dehydrogenase, a host cell transformed with such a vector, and a method for preparing the engineered copper dehydrogenase by culturing the transformant, collecting and purifying the engineered copper dehydrogenase from the culture.

IV. Devices

L-DOPA Biosensors

[0263] In another embodiment, a device for assaying L-DOPA in a sample is provided, where the device includes an engineered copper dehydrogenase as described herein.

[0264] Disclosed herein is a L-DOPA biosensor comprising an engineered copper dehydrogenase used to measure physiologically relevant L-DOPA concentrations as shown in **Figure 2**. In some embodiments, the engineered copper dehydrogenase is a L-DOPA dehydrogenase enzyme, wherein the enzyme has improved substrate specificity for L-DOPA over biologically relevant phenols, dopamine analogues, and sugars. Described herein, L-DOPA is measured utilizing electrochemical techniques. In some embodiments, the L-DOPA biosensor measures L-DOPA through open-circuit potential, transient open circuit potential, chronoamperometry, square-wave voltammetry, and/or extended gate field-effect transistor (EGFET) detection, or a combination of these methods. In one example, several methods such as chronoamperometry, open-circuit potential, and transient open-circuit potential will be used cyclically in a specific protocol for continuous, intermittent, or single point measurements (**Figures 26A and 26B**).

[0265] In embodiments provided herein, the L-DOPA biosensor provides for sensitive and specific detection of L-DOPA.

[0266] In some embodiments, the engineered copper dehydrogenase is immobilized onto an electrode. In some embodiments, the electrode is a disk electrode or a needle or wire electrode. In one embodiment the electrode is a gold disk rod. In another embodiment the electrode is a gold wire or needle type electrode. In some embodiments the L-DOPA biosensor has a three-electrode configuration comprising a working electrode (WE), a reference electrode (Ref), and a counter electrode (CE). In some embodiments the L-DOPA biosensor has a two-electrode configuration comprising a WE and a shared Ref/CE. *See Figure 2*. The concentration of L-DOPA in a sample may be determined by measuring the amount of electrons generated by the enzyme reaction. In embodiments, a sensor system may comprise a gold (Au) electrode, a silver chloride (Ag/AgCl) electrode, a platinum (Pt) electrode, a carbon (C) electrode, and/or another metal electrode.

[0267] In one embodiment, a sample of non-biological derived or biological derived, such as a blood, a serum, a saliva, a tear, a urine, a sweat or interstitial fluid sample, is added to the sample receiver, L-DOPA contained in the sample will react with the engineered copper dehydrogenase, transferring electrons to the electrode to generate a current, which is indicative of the amount of L-DOPA in the sample.

[0268] In one embodiment, the engineered copper dehydrogenase can be immobilized on electrodes. Examples of means for immobilizing molecules such as the engineered MCO include,

but are not limited to, cross-linking, encapsulating into a macromolecular matrix, coating with a dialysis membrane, optical cross-linking polymer, self-assembled monolayer, electroconductive polymer, oxidation-reduction polymer, and any combination thereof.

[0269] In some embodiments, the sensor further comprises an outer membrane. In some embodiments, the outer membrane comprises cellulose acetate, poly(ethylene glycol) diglycidyl ether (PEGDE), polyethylene glycol (PEG), polyurethane, an epoxy resin, or Nafion. In one example various weight to volumes of cellulose acetate may be added to the electrode, through drop casting or dip coating (**Figures 27A-27D, 28A-28D, and 29A-29B**). The electrode may or may not be pretreated in solvent to enhance the membrane formation.

[0270] In embodiments, the electrode is a screen-printed carbon electrode, a planar gold electrode, or an interdigitated electrode array. When the measurement is conducted in an amperometric system using a carbon (C) electrode, gold (Au) electrode or platinum (Pt) electrode provided with an immobilized enzyme is used as a working electrode, together with a counter electrode (such as a Pt electrode) and a reference electrode (such as an Ag/AgCl electrode). The electrodes can be inserted into a buffer containing a mediator, or have a sample deposited onto the electrode with, or without mediator and kept at predetermined temperature.

[0271] A predetermined voltage can be applied to the working electrode with respect to a reference electrode, and then a sample is added and an increased value in electric current is measured. It is generally also possible to use so-called two-electrode systems with one working electrode and one counter or pseudo-reference electrode.

[0272] In one embodiment, the working electrode can be inserted into buffer together with a counter electrode (such as a Pt electrode) and a reference electrode (such as an Ag/AgCl electrode) and kept at a predetermined temperature. As indicated above, a predetermined voltage can be applied to the working electrode, and then the sample is added and increased value in electric current is measured.

[0273] In some embodiments, the L-DOPA biosensor can continuously assay L-DOPA in a sample.

[0274] In some embodiment, the engineered copper dehydrogenase is immobilized on a sensor strip, which may be screen-printed, or spattered or fabricated in any other manner known in the art. In embodiments, the sensor strips may be single-use and/or disposable. L-DOPA sensor strips can be used for point-of-care testing (POCT) and for personal use L-DOPA monitoring.

[0275] Also provided herein are biosensors for the detection of foslevodopa ((2S)-2-amino-3-(3-hydroxy-4-phosphonooxyphenyl)propanoic acid, dopa 4-phosphate, or levodopa-4'-monophosphate), a medication for the treatment of patients with PD. The sensors comprise a copper dehydrogenase described above and further comprise an enzyme to catalyze the dephosphorylation of foslevodopa to L-DOPA. In some embodiments, the enzyme is a phosphatase. In some embodiments, the enzyme that catalyzes the phosphorylation of foslevodopa is immobilized as a layer on the working electrode. In some embodiments, the enzyme that catalyzes the phosphorylation of foslevodopa is provided in solution in the device. Once the foslevodopa has been dephosphorylated by the enzyme (e.g., phosphatase), the copper dehydrogenase sensor is used to detect the resulting L-DOPA product as previously described. Thus, a device comprising both enzymes can be used to detect foslevodopa.

[0276] Accordingly, these devices can be used as continuous foslevodopa biosensors for point of care testing (POCT) for PD patients treated with foslevodopa. The foslevodopa monitoring systems disclosed herein have the potential to significantly enhance the management of stage II PD and minimize “off-time” PD symptoms.

Enzyme Fuel Cells

[0277] Disclosed herein, isolated copper dehydrogenases are used to catalyze the oxidation of an immobilized substrate at the anode of an enzyme fuel cell. One challenge with existing enzyme fuel cells which utilize physiological substrates in solution is the availability of the enzyme's substrate to power the fuel cell. Provided herein are enzyme fuel cells utilizing an engineered copper dehydrogenase to catalyze the oxidation of an immobilized substrate at the anode. The immobilized substrate at the anode is the fuel source for the enzyme fuel cell and the oxidation of the substrate allows for electrons to be transferred to the anode. The enzyme fuel cells disclosed herein are provided with a fuel source which is consistently available at the point of the reaction, allowing for the enzyme fuel cell to remain at maximum power throughout the duration of its operational lifespan. The design of an enzyme fuel cell with an enzyme immobilized with its substrate on the anode as disclosed herein provides practical advantages over previous enzyme fuel cells which rely on the availability of a substrate in solution.

[0278] The term “substrate” refers to a molecule which is oxidized during the enzymatic reaction of the anode. The term “physiological substrate” refers to a biological molecule such as a

sugar or metabolite (e.g., glucose, fructose, lactate, cholesterol, etc.) which is oxidized during an enzymatic reaction.

[0279] The term “immobilized” refers to a compound which is attached to a surface (e.g., electrode). Immobilization can be performed through adsorption, cross-linking, covalent bonding and/or affinity-tag binding.

[0280] The term “cross-linking” refers to a polymerization reaction of a cross-linking reagent. A “cross-linking reagent” refers to a molecule that contains two or more reactive ends capable of forming covalent bonds.

[0281] In some embodiments, the fuel source immobilized on the electrode is formed by depositing a cross-linking reagent. In some embodiments, the cross-linking reagent comprises aldehyde functional groups. In some embodiments, the cross-linking reagent is glutaraldehyde. Glutaraldehyde may react with basic amino acid residues (e.g., lysine) of a protein or enzyme (e.g., multicopper oxidase and/or BSA deposited on an electrode). There is no general scientific agreement on the reactive species of glutaraldehyde, which can form many variable intermediates and products during the cross-linking process, which are not well characterized. Without wishing to be bound by theory, it is believed that the cross-linking of glutaraldehyde forms phenolic compounds, which can be oxidized as a fuel source by multicopper oxidase enzymes to power enzyme fuel cells.

[0282] Engineered copper dehydrogenases disclosed herein as anode catalysts for enzyme fuel cells can oxidize various substrates, such as phenolic compounds. In embodiments, the fuel source immobilized on the anode is a phenolic compound.

[0283] The term “phenolic compound” refers to a molecule comprising phenol (hydroxybenzene). A phenolic compound can be a polymer formed from the cross-linking of glutaraldehyde. Phenolic compounds can be large molecules with additional functional groups and can include multiple phenol moieties, such as lignin.

[0284] In some embodiments, the phenolic fuel source is derived from lignin. It may be desired with respect to enzyme fuel cells to use a sustainable and non-toxic fuel source. Lignin, a class of phenolic polymers naturally produced by a variety of plants and biomass, is a plentiful, renewable, and non-hazardous material, making it a desirable fuel source for an enzyme fuel cell.

[0285] Engineered copper dehydrogenases disclosed herein as anode catalysts for enzyme fuel cells can oxidize various substrates, such as phenolic compounds. In embodiments, the fuel source immobilized on the anode is a phenolic compound.

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[0287] In some embodiments, the phenolic fuel source is derived from lignin. It may be desired with respect to enzyme fuel cells to use a sustainable and non-toxic fuel source. Lignin, a class of phenolic polymers naturally produced by a variety of plants and biomass, is a plentiful, renewable, and non-hazardous material, making it a desirable fuel source for an enzyme fuel cell.

[0288] Disclosed herein are enzyme fuel cells utilizing an engineered copper dehydrogenase and a substrate (e.g., fuel source) immobilized on an anode, providing a constant source of fuel and power. The enzyme catalyzes the oxidation of a substrate (e.g., dehydrogenation of phenol), transferring electrons to the anode, while the cathode reduces oxygen. The cathode is in a solution (e.g. aqueous) with dissolved oxygen. Ambient oxygen from a physiological environment is sufficient to catalyze the cathode half reaction.

[0289] Disclosed herein, enzyme fuel cells comprise an electron acceptor on the cathode to reduce oxygen. In some embodiments, the electron acceptor comprises an oxidase enzyme. In embodiments, electron acceptors can be prepared by conjugating an electron acceptor molecule to a protein or enzyme. In some embodiments, the conjugation can be performed with an amine reactive moiety (e.g., NHS ester).

[0290] In other embodiments the electron acceptor is a molecule other than an enzyme. In some embodiments, the electron acceptor is a phenazine compound.

[0291] The term “phenazine compound” refers to a molecule comprising a 9,10-diazaanthracene moiety. A phenazine compound may be a salt.

[0292] In some embodiments, the phenazine compound comprises phenazine ethosulfate (PES) or phenazine methosulfate (PMS). In some embodiments the phenazine compound is amine-reactive.

[0293] The term “amine-reactive” refers to a chemical group which will react with a primary amine moiety (e.g., lysine residue). Amine-reactive chemical groups include N-hydroxysuccinimide esters (NHS ester) and imidoesters.

[0294] In some embodiments the amine-reactive phenazine compound is N-hydroxysuccinimidylester 1-propoxy-5-ethylphenazinium ethyl sulfate (arPES).

[0295] In other embodiments, the electron acceptor comprises a metal complex. In some embodiments the metal complex is an osmium complex or a ruthenium complex.

[0296] In some embodiments, the electron acceptor is immobilized on a protein. In some embodiments the protein is bovine serum albumin (BSA), glucose dehydrogenase (GDH), lactate dehydrogenase (LDH), glucose oxidase (GOx), and/or lactate oxidase (LOx).

[0297] The cathode and anode of the enzyme fuel cell provided herein comprise an electroconductive material. In some embodiments, the electroconductive material comprises carbon paper, glassy carbon, carbon nanotubes, gold, and/or palladium. In some embodiments, the cathode and/or anode comprises conductive ink.

[0298] The term “electroconductive material” refers to a substance capable of transmitting electricity.

[0299] The engineered copper dehydrogenase catalyzing the anode reaction, the electron acceptor of the cathode (e.g., phenazine compound, metal complex or enzyme), and the substrate (e.g., fuel source) are immobilized on the electrodes. The immobilization may be performed by any method known in the art (e.g., cross-linking with glutaraldehyde). In some embodiments, the substrate (e.g., fuel source) may be formed through the cross-linking process (e.g., phenolic compounds formed by the cross-linking of glutaraldehyde). In some embodiments, another substrate may be provided in addition to the cross-linking reagent (e.g., lignin-derived materials).

[0300] In some embodiments, another protein may be immobilized on the anode in addition to the engineered copper dehydrogenase enzyme (e.g., BSA). In some embodiments, a protein may be immobilized on the cathode in addition to the electron acceptor (e.g., phenazine compound, metal complex, or oxidase enzyme). In some embodiments, the enzyme, substrate, electron acceptor, and/or protein of the anode and/or cathode may be immobilized in a hydrogel.

[0301] In embodiments, the anode and the cathode are in a solution, which may contain electrolytes. In some embodiments, the anode and cathode are in the same environment. The solution containing the cathode comprises dissolved oxygen, which may be ambient oxygen from

physiological conditions. In other embodiments the anode and cathode are in separate environments and are connected by a salt bridge. The salt bridge can be composed of any material known in the art.

BioBattery

[0302] A BioBattery is provided comprising any of the enzyme fuel cells disclosed herein. The BioBattery comprises an enzyme fuel cell provided herein for the consistent generation of power from an oxidoreductase enzyme catalyzing the oxidation of a substrate immobilized on an anode while ambient oxygen is reduced at a cathode. The anode may comprise any of the engineered copper dehydrogenases, fuel sources, and electroconductive materials disclosed herein immobilized on an electrode. The cathode may comprise any of the electron acceptors immobilized on an electrode as disclosed herein (e.g., phenazine compounds, metal complexes, or oxidase enzymes) to reduce oxygen.

[0303] The BioBattery disclosed herein comprises an anode and a cathode of said enzyme fuel cell enclosed in a container. In some embodiments, the BioBattery may comprise a salt bridge between the anode and the cathode. The BioBattery may be contained by any material known in the art. In some embodiments the BioBattery comprises a biodegradable material. A BioBattery comprising a biodegradable material disclosed herein has the advantage of environmental sustainability and reduced toxicity compared to existing batteries.

[0304] The term “biodegradable material” refers to a substance which can be decomposed by microorganisms through composting or by normal environmental processes.

[0305] It may be desired with respect to a battery to modulate power and current output for powering biomedical devices. The design of the BioBattery disclosed herein allows for modification of the number of enzyme fuel cells and the configuration connecting multiple fuel cells to adjust power and current output based on the needs of the biomedical device.

[0306] In some embodiments the BioBattery comprises one enzyme fuel cell disclosed herein. In other embodiments, the BioBattery comprises more than one enzyme fuel cell disclosed herein. Multiple enzyme fuel cells can be stacked to increase the current output. In some embodiments, multiple enzyme fuel cells are connected in series configurations. In other embodiments, multiple enzyme fuel cells are connected in parallel configurations. In embodiments, batteries comprising multiple stacked enzyme fuel cells can be used to power biomedical devices.

V. Kits

[0307] In another embodiment, a kit for assaying L-DOPA in a sample, where the kits include at least an engineered copper dehydrogenases as described herein.

[0308] Additionally, the kits can include a buffer necessary for the measurement, a standard solution of L-DOPA for preparing a calibration curve and/or an instruction for use. The engineered copper dehydrogenase may be provided in various forms such as, for example, a freeze-dried reagent or a solution in an appropriate storage solution.

[0309] In some embodiments, the kit includes sensor strips, which may be screen-printed and/or disposable.

[0310] Any or all of the kit reagents can be provided within containers that protect them from the external environment, such as in sealed containers. Positive and/or negative controls can be included in the kits to validate the activity and correct usage of reagents employed in accordance with the inventive concept. Controls can include samples known to be either positive or negative for the presence of a predetermined concentration of L-DOPA.

VI. Methods

[0311] The engineered copper dehydrogenases disclosed herein can be used in various methods. For example, they can be used in methods of assaying L-DOPA in a sample from a subject. In embodiments, the sample comprises material selected from the group consisting of blood, serum, saliva, tears (i.e., lacrimal gland secretions), urine, sweat, and interstitial fluid.

[0312] The method can include at least a step of contacting the sample with the engineered copper dehydrogenase and a step of measuring the amount of the L-DOPA oxidized by the engineered copper dehydrogenase as described above and further below. In embodiments, the method includes continuous measurement of the amount of L-DOPA oxidized by the engineered copper dehydrogenase.

[0313] In some embodiments, the copper dehydrogenases provided herein are used to detect L-DOPA derived from foslevodopa (((2S)-2-amino-3-(3-hydroxy-4-phosphonooxyphenyl)propanoic acid, or dopa 4-phosphate, levodopa-4'-monophosphate). The foslevodopa in the sample from the subject is first dephosphorylated to produce L-DOPA. In some embodiments, the L-DOPA assayed by the method is derived from foslevodopa, and the method further comprises contacting the sample with an enzyme capable of dephosphorylating

foslevodopa to L-DOPA before contacting the sample with copper dehydrogenase. Phosphatase enzymes can be used for the dephosphorylation. The resulting L-DOPA is then quantified by the device comprising the copper dehydrogenase as described.

[0314] These methods may be adapted, *mutatis mutandis*, for the assay of other substrates modified by the engineered enzymes disclosed herein.

[0315] The disclosed subject matter is further described in the following non-limiting Examples. It should be understood that these Examples, while indicating preferred embodiments of the subject matter, are given by way of illustration only.

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EXAMPLES

*Example 1A: Modifying a *Pyrobaculum aerophilum* MCO for reduced oxidase activity*

[0316] A *Pyrobaculum aerophilum* derived multicopper oxidase (PaMCO) mutant harboring Phe290Ile was chosen as a representative case for engineering to reduce oxidase activity and improve biosensing capabilities. The structural gene pae1888 encoding PaMCO was inserted into a pET-11a expression vector (SEQ ID NO: 17) and mutated based on conventional methods using oligonucleotides (**Figure 23**). Recombinant engineered PaMCOs were prepared using *Escherichia coli* as a host microorganism and purified using anion exchange chromatography.

[0317] Recombinant engineered PaMCOs were produced with the amino acid substitutions shown in **Table 1**, and the mutations were confirmed by nucleotide sequencing. PaMCO mutants were successfully produced as soluble proteins and purified, showing expected molecular weights (**Figure 3**).

Table 1: Amino acid substitutions of *Pyrobaculum aerophilum* multicopper oxidase mutants

Sequence	Amino Acid Substitution(s)
SEQ ID NO: 1	His94Ala/Phe290Ile
SEQ ID NO: 2	His132Ala/Phe290Ile
SEQ ID NO: 3	Phe290Ile/His396Ala
SEQ ID NO: 4	Phe290Ile/His459Ala
SEQ ID NO: 5	Phe290Ile/His396Ala/His459Ala
SEQ ID NO: 6	His94Ala/Phe290Ile /His396Ala/His459Ala
SEQ ID NO: 7	Phe290Ile

[0318] Oxidase activities of the MCO mutants were determined using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as a substrate by monitoring the increase in absorbance at 420nm based on the formation of reduced ABTS. **Figure 4** shows the results of oxidase activity measurement of each PaMCO mutant. These results indicate that all mutant PaMCOs (SEQ ID NOs: 1-6) repressed their oxidase activity towards oxygen to less than 10% of the initial PaMCO Phe290Ile enzyme (SEQ ID NO: 7). Mutant PaMCOs comprising His94Ala (SEQ ID NO: 1), His132Ala (SEQ ID NO: 2), His396Ala (SEQ ID NO: 3), and

His396Ala/His459Ala (SEQ ID NO: 5) and His94Ala/His396Ala/His459Ala (SEQ ID NO: 6) all repressed oxidase activity towards oxygen to less than 1.5% of the initial PaMCO Phe290Ile enzyme (SEQ ID NO: 7). These results indicate that the mutation of residues His94, His132, His396, His459, which are T2 and T3 copper-binding ligands, repress PaMCO oxidase activity towards oxygen.

[0319] Spectroscopic analysis of PaMCO and its mutants (**Figure 50**) confirms the presence of the Cu atom at the T1 binding center in the mutant PaMCO. Similar to the wild type PaMCO, the typical absorption spectrum around 620 nm of the T1 Cu in PaMCO was observed. These results indicate that the mutant PaMCOs retain the Cu atom at the T1 Cu binding site. Thus, the mutant PaMCO enzymes with depressed oxidase activity show dye-mediated dehydrogenase activity, keeping DET-ability with electrode.

Example 1B: Modifying an Escherichia coli multicopper oxidase for reduced oxidase activity

[0320] An *Escherichia coli* multicopper oxidase (WP_166488460, UniProtID:P36649, CueO SEQ ID NO: 19) was chosen as a representative case for engineering to reduce oxidase activity and improve biosensing capabilities. The structural gene encoding CueO was inserted into a pET-11a expression vector and mutated based on conventional methods using oligonucleotides. Recombinant engineered CueO were prepared using *Escherichia coli* as a host microorganism and purified using anion exchange chromatography.

[0321] Recombinant engineered CueO were produced with the amino acid substitutions, and the mutations were confirmed by nucleotide sequencing. CueO mutants, H448A (single mutant), H448A/H498A (double mutant), H103A/H448A/H498A (triple mutant), were successfully produced as soluble proteins and purified, showing expected molecular weights.

[0322] Oxidase activities of the CueO mutants were determined using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as a substrate by monitoring the increase in absorbance at 420nm based on the formation of reduced ABTS. **Figures 52A-52B** shows the results of oxidase activity measurement of each CueO mutant. These results indicate that all mutant CueO repressed their oxidase activity towards oxygen to less than 10% of the initial wild type CueO (SEQ ID NO: 19). Mutant CueOs comprising H448A (single mutant), H448A/H498A (double mutant), H103A/H448A/H499A (triple mutant) all repressed oxidase activity towards oxygen to less than 1.0% of the initial CueO enzyme (SEQ ID NO: 19). These results indicate that

the mutation of residues His103, His448, His498, which are T2 and T3 copper-binding ligands, repress CueO oxidase activity towards oxygen.

[0323] Spectroscopic observation of CueO and its mutants (**Figure 53**) confirms the presence of the Cu atom at the T1 binding center in the mutant CueO, similar to the wild type CueO, PaMCO and its mutants (Figure 50), exhibiting the typical absorption spectrum around 620 nm of the T1 Cu in CueOs. These results indicate that the mutant CueOs also retain the Cu atom at the T1 Cu binding site to provide dehydrogenase activity and DET-ability with an electrode.

Example 1C: Modifying a *Bacillus subtilis* Laccase for reduced oxidase activity

[0324] A *Bacillus subtilis* Laccase (CotA, UniProtID:P07788, SEQ ID NO: 20) was chosen as a representative case for engineering to reduce oxidase activity and improve biosensing capabilities. The structural gene encoding CotA is inserted into a pET-11a expression vector and mutated based on conventional methods using oligonucleotides. Recombinant engineered CotA are prepared using *Escherichia coli* as a host microorganism and purified using anion exchange chromatography.

[0325] Recombinant engineered CotAs are produced with the amino acid substitutions, and the mutations are confirmed by nucleotide sequencing. CotA mutant, His422Ala was successfully produced as a soluble protein and purified, showing expected molecular weights (Figure 53).

[0326] The oxidase activity of the CotA mutant was determined using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as a substrate by monitoring the increase in absorbance at 420nm based on the formation of reduced ABTS. **Figure 54** shows the results of oxidase activity measurement of the CotA His422Ala mutant and the wild type. These results indicate that all mutant CotA repressed their oxidase activity towards oxygen to less than 10% of the initial wild type CotA (SEQ ID NO: 20). These results indicate that the mutation of residue His422, which is a T2 copper-binding ligands, represses CueO oxidase activity towards oxygen.

Example 2: A L-DOPA biosensor utilizing an engineered copper dehydrogenase for improved L-DOPA dehydrogenase activity

[0327] Electrodes were prepared with immobilized PaMCOs. Gold disk electrodes were polished with 0.3 and 0.05 μ M alumina powder and then soaked in a piranha solution consisting

of a 3:1 sulfuric acid to hydrogen peroxide solution for one hour. Afterwards, the electrodes were electrochemically cleaned using cyclic voltammetry in a solution of 50 mM KOH, sweeping between 0V-1.2 V. Afterwards, electrodes were washed with acetone and then incubated overnight in 100 μ M dithiobis(succinimidyl hexanoate) self-assembled monolayer (DSH-SAM) solution at 25°C, 300 rpm. Next, electrodes were incubated overnight in 0.014 mg/ml solution of the PaMCO enzyme at 25°C, stirring at 300 rpm. Finally, electrodes were washed by 100 mM potassium phosphate buffer (PPB) and then stored in 100 mM PPB until use. Electrodes were prepared with the initial PaMCO Phe290Ile enzyme (SEQ ID NO: 7) and the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant enzyme (SEQ ID NO: 5).

[0328] Electrical current was measured for both PaMCO electrodes in the presence of L-DOPA as the substrate. As shown in **Figure 5** and **Figure 37**, the initial PaMCO Phe290Ile enzyme (SEQ ID NO: 7) had a much lower L-DOPA current response in the presence of oxygen, indicating a lower dehydrogenase activity when compared to the engineered PaMCO His396Ala/His459Ala/Phe290Ile mutant enzyme (SEQ ID NO: 5). The design of the engineered PaMCO His396Ala/His459Ala/Phe290Ile mutant enzyme (SEQ ID NO: 5) has enabled this enzyme to dramatically improve its dehydrogenase activity (e.g., towards L-DOPA) while also lowering its oxidase activity towards oxygen. The engineered PaMCO mutant enzyme is shown to be an oxygen insensitive copper dehydrogenase, specifically a L-DOPA dehydrogenase (L-DOPADh) capable of using L-DOPA as a substrate. The L-DOPA dehydrogenase immobilized onto an electrode is capable of acting as L-DOPA biosensor in the presence of oxygen.

[0329] Further testing of the L-DOPA biosensor was performed. Chronoamperometric measurements showed that the L-DOPA biosensor employing the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant enzyme (SEQ ID NO: 5) was able to accurately sense L-DOPA concentrations in the 0-60 μ M range tested. **Figure 6A** shows amperometric raw data and **Figure 6B** shows an amperometric calibration curve of the L-DOPA biosensor with a RSD value of 0.9918. Amperometric testing was further evaluated with a range of applied voltage between 0.2 V up to 0.7 V, showing linear L-DOPA response over the therapeutic relevant range (**Figures 38A-38G**).

[0330] Additionally, open circuit potential measurements of the L-DOPA biosensor employing the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant enzyme (SEQ ID NO: 5) provided accurate detection of L-DOPA concentrations. **Figure 7A** shows open circuit

potential raw data and **Figure 7B** shows an open circuit potential calibration curve of the L-DOPA biosensor with a RSD value of 0.9933.

[0331] Next, square wave voltammetry measurements of the L-DOPA biosensor employing the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant enzyme (SEQ ID NO: 5) showed that the mutant enzyme allowed for concentration-dependent detection of L-DOPA. **Figure 8A** shows square wave voltammetry raw data and **Figure 8B** shows a square wave voltammetry calibration curve of the L-DOPA biosensor with a RSD value of 0.98. Various frequencies were applied, ranging from 1 – 1000 mV/sec, displaying two distinct peaks. **Figures 47A-47C** show results, displaying 3 examples frequencies of 1, 100, and 1000 mV/sec, as well as **Figures 47D-F** displaying the slope (response of Δ current in μ A to L-DOPA concentration) and Pearson Correlation (RSQ) across the entire frequency range tested with respect to L-DOPA concentration.

[0332] Furthermore, extended gate field-effect transistor (EGFET) measurements of the L-DOPA biosensor employing the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant enzyme (SEQ ID NO: 5) were also able to detect L-DOPA in a concentration-dependent manner. **Figure 9A** shows EGFET raw data and **Figure 9B** shows a EGFET calibration curve of the L-DOPA biosensor with a RSD of 0.98.

[0333] Thus, experimental results indicate that the L-DOPA biosensor employing an engineered PaMCO is capable of accurately sensing L-DOPA concentrations in various solutions through several different of measurement methods including chronoamperometry, open-circuit potential, square-wave voltammetry, and extended gate field-effect transistor (EG-FET) detection. A challenge for the utility of L-DOPA biosensors is having a sufficiently low limit of detection at biologically relevant concentrations, concentration range of L-DOPA is 0.5-15 μ M in plasma and 0.1-2.5 μ M in sweat. Many reported technologies often test outside of this range which presents significant issues for biosensing applications. The engineered PaMCO mutants described herein overcome this difficulty by accurately measuring L-DOPA at sufficiently low limits of detection, well within the physiologically relevant L-DOPA concentration range.

Example 3: The L-DOPA biosensor has minimal interferent impact towards physiologically relevant compounds, and environmental changes

[0334] Additional testing of the L-DOPA biosensor employing the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant enzyme (SEQ ID NO: 5) showed ideal substrate specificity for L-DOPA over other physically relevant compounds including quinones, sugars, phenols, and carbidopa (C-DOPA).

[0335] The L-DOPA biosensor employing the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant enzyme (SEQ ID NO: 5) was tested with the addition of C-DOPA across various L-DOPA concentration profiles. **Figure 10** shows the open circuit potential bias from 1-60 μ M of L-DOPA, indicating limited bias from the addition of C-DOPA. **Figure 11** shows the chronoamperometry signal bias from the addition of C-DOPA across various L-DOPA concentration profiles, also exhibiting minimal bias.

[0336] The enzyme specificity for L-DOPA over C-DOPA is highly advantageous for a biosensor for managing medication dosing for PD patients. PD treatment usually involves the administration of both L-DOPA as a dopamine precursor and C-DOPA as decarboxylase inhibitor to prevent L-DOPA from being broken down before it reaches the brain. Thus, PD patients in need of L-DOPA monitoring will likely also have C-DOPA in their systems. Existing biosensors which are non-specific and incapable of distinguishing between L-DOPA and C-DOPA will therefore not be able to accurately detect L-DOPA levels in PD patients. The engineered MCOs disclosed herein overcome this limitation with their L-DOPA specificity and therefore provide great potential for improved L-DOPA detection for PD patients.

[0337] Additionally, the L-DOPA biosensor employing the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant enzyme (SEQ ID NO: 5) was tested under conditions with various physiologically relevant compounds that including methyldopa, dopamine, chlorthalidone, amantadine, and acetaminophen. The abovementioned molecules did not show significant signal bias in the presence of 10 μ M L-DOPA at high end physiological concentrations as shown by chronoamperometric (**Figure 12**) and open circuit potential (**Figure 13**) measurements, which improved by modulating the applied voltage. The same electrodes were tested at various applied potentials to investigate interferent impact.

[0338] Additionally, the signal bias of a L-DOPA sensor using tyrosinase was compared to the L-DOPA sensor using the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant

enzyme. All measurements were taken with specific physiologically relevant concentrations of the interferents which were added to a solution containing 180 μM L-DOPA. Previous L-DOPA sensors employed in the literature have used tyrosinase, which exhibited significantly higher signal bias in the presence of physiological interferents relevant to Parkinson's disease patients (**Figure 14**) as compared to the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant enzyme (**Figure 15**, **Figure 33A-33B**, and **Figure 44A-44C**). These surprising and encouraging results suggest that copper dehydrogenases engineered for reduced oxidase activity are more selective and accurate for the detection of L-DOPA than previously used enzymes, such as tyrosinase.

[0339] Additionally, L-DOPA sensors employing the same electrochemical algorithm were used in **Figure 26A** for the interference evaluation. **Figures 39A, 39D, and 39G** illustrate the impacts of modulating 50-150 mM KCl on the chronoamperometric, open-circuit potential, and transient open-circuit potential signals, respectively, in a background of 10 μM levodopa solution. **Figures 39B, 39E, and 39H** display how changing the pH (6.0-8.0) of a 100 mM PPB solution affects the chronoamperometric, open-circuit potential, and transient open-circuit potential signals, respectively, when measuring 10 μM levodopa. **Figures 39C, 39F, and 39I** highlight the variations in the chronoamperometric, open-circuit potential, and transient open-circuit potential signals, respectively, when measuring levodopa concentrations of 1-55 μM in 100 mM PPB (pH 7.0) at room temperature versus body temperature.

Example 4: L-DOPA biosensors can be fabricated utilizing various types of enzyme immobilization techniques

[0340] Various methods were tested for immobilization of the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant enzyme (SEQ ID NO: 5) onto the electrode, including glutaraldehyde, poly(3,4-ethylenedioxythiophene) polystyrene sulfonate (PEDOT:PSS), copper sulfate nanoflower, DSH-SAM, and Nafion. For all immobilization techniques tested, gold disk electrodes were polished using 0.3 and 0.05 μm aluminum powder followed by soaking in piranha (3:1 sulfuric acid to hydrogen peroxide) for 1 hour. After soaking for 1 hour, each electrode was electrochemically cleaned using cyclic voltammetry in a solution of 50 mM KOH, sweeping between 0 V-1.2 V.

[0341] For glutaraldehyde immobilization, 7 μL of a 6.3 mg/mL solution of the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant enzyme (SEQ ID NO: 5) was drop casted onto

the electrode and allowed to dry in at room temperature in an incubator. After drying, the electrode was crosslinked by vapor depositing a 25% glutaraldehyde solution for 1 hour. After crosslinking the electrode was then placed in 10 mM Tris-HCl buffer for 20 minutes, and then stored in 100 mM PPB until use. An amperometric current response of a L-DOPA biosensor fabricated by enzyme immobilization with glutaraldehyde is shown in **Figure 16A** with an RSD value of 0.99.

[0342] For PEDOT immobilization, gold disk electrodes were polished using 0.3 and 0.05 μm aluminum powder and electrochemically cleaned using cyclic voltammetry in a solution of 50 mM KOH, sweeping from 0 - -1.2 V. Enzyme immobilization occurred through drop casting 5 μL of the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant enzyme (SEQ ID NO: 5) at a concentration of 1, 2, and 3 mg/mL and 1% PEDOT:PSS at a ratio of 1:1. The electrode was allowed to dry at room temperature in an incubator and stored in 100 mM potassium phosphate buffer until further testing. An amperometric current response of a L-DOPA biosensor fabricated by enzyme immobilization with the three different concentrations of PEDOT:PSS is shown in **Figure 16B**.

[0343] For copper sulfate nanoflower immobilization, gold disk electrodes were polished using 0.3 and 0.05 μm aluminum powder followed by soaking in piranha (3:1 sulfuric acid to hydrogen peroxide) for 1 hour. After soaking, each electrode was electrochemically cleaned using cyclic voltammetry in 50 mM KOH solution, sweeping from 0 - -1.2 V. 100 μM DSH was then immobilized using acetone overnight on a shaker at 25°C at 300 rpm. Following this incubation, 0.014 mg/mL of the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant enzyme (SEQ ID NO: 5) was immobilized overnight 25°C at 300 rpm, then stored in 100 mM potassium phosphate buffer until further testing. Then, the electrodes were incubated in 200 microliters of a copper sulfate nanoflower solution overnight at 25°C at 300 rpm. An open-circuit potential response of a L-DOPA biosensor fabricated by enzyme immobilization with copper sulfate nanoflowers is shown in **Figure 16C**, with an RSD value of 0.98. Additionally, an amperometric, open-circuit potential, and transient open circuit potential sensor was fabricated using the electrochemical protocol described as shown in **Figures 48A-G** using 3 replicates and displaying strong L-DOPA dependent signal.

[0344] For DSH-SAM immobilization, gold disk electrodes were polished using 0.3 and 0.05 μm aluminum powder followed by soaking in piranha (3:1 sulfuric acid to hydrogen peroxide) for 1 hour. After soaking, each electrode was electrochemically cleaned using cyclic voltammetry

in 50 mM KOH solution, sweeping from 0 - -1.2 V. 100 or 500 μ M DSH was then immobilized using acetone overnight on a shaker at 25°C at 300 rpm. Following this incubation, 0.014 mg/mL of the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant enzyme (SEQ ID NO: 5) was immobilized overnight at 25°C at 300 rpm, then stored in 100 mM potassium phosphate buffer until further testing. An amperometric current response of a L-DOPA biosensor fabricated by enzyme immobilization with DSH-SAM is shown in **Figure 16D**, with an RSD value of 0.9907. Additionally, using either 100 or 500 μ M DSH an amperometric, open-circuit potential, and transient open circuit potential sensor was fabricated using the electrochemical protocol described in **Figure 26A**. Either 0.3 V or 0.4 V was applied during chronoamperometry, and all amperometric, open-circuit potential, and transient open circuit potential gave L-DOPA dependent response across the therapeutic window as shown in **Figures 41A-41F**.

[0345] For Nafion immobilization, 7 μ L of a 6.3 mg/mL solution of the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant enzyme (SEQ ID NO: 5) was drop casted onto the electrode and allowed to dry in at room temperature in an incubator. Then, 7 μ L of 1% Nafion was dropcast over the enzyme and dried in an incubator, then electrodes were washed with 100 mM PPB and stored in 100 mM PPB until use. An amperometric current response of a L-DOPA biosensor fabricated by enzyme immobilization with Nafion is shown in **Figure 16E** with an RSD value of 0.9977.

Example 5: A L-DOPA biosensor that can be miniaturized into a smaller implantable or wearable formfactor

[0346] In some embodiments the engineered copper dehydrogenase is immobilized onto the wire electrode, made of up gold, platinum, stainless steel, or carbon. This electrode will have either a Ref, and CE electrode in a 3-electrode system, or will have the Ref/CE shorted in a two electrode system. The wire electrode may range in length from 3 – 25 mm, with a working electrode radius from 10 – 250 μ m, a Ref electrode radius from 10 – 250 μ m, and a CE electrode radius from 10 - 250 μ m. These electrodes will be joined adjacently using insulation materials, including polyimide, poly-vinyl chloride, poly-vinyl alcohol, polytetrafluoroethylene (PTFE), polyethylene (PE), polyetheretherketone (PEEK), perfluoroalkoxy (PFA). **Figure 30** shows an example of one embodiment, having a 76 μ m gold, working electrode, with the shared Ref/Ce wrapped around. The Ref/CE to WE surface area ratio may be between 1 – 15. In some

embodiments the WE electrode is roughened or altered to increase the apparent surface area. In one example, this surface area enhancement may be 2 – 3 times greater post surface alteration (**Figure 43**).

[0347] To prepare a miniaturized L-DOPA biosensor, a sander, straight blade, or etching was used to remove the PFA coating on a purchased gold nanowire. Afterwards, the gold nanowire was cleaned with sodium hydroxide (NaOH), or potassium hydroxide via cyclic voltammetry sweeps. Then, the functional surface area was determined, and the gold nanowire was soaked in a dithiobis(succinimidyl hexanoate) (DSH) solution, followed by immobilization of the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant enzyme (SEQ ID NO: 5). A reference electrode was fabricated by electrodepositing Ag/AgCl in 1M KCl at pH 3.5 and was stored overnight in 1M KCl.

[0348] The miniaturized L-DOPA biosensor was tested for the detection of L-DOPA at various concentrations. **Figure 17** shows the chronoamperometric response and of the needle-type L-DOPA biosensor employing the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant enzyme (SEQ ID NO: 5). **Figure 18** shows the open circuit potential response of the needle or wire-type L-DOPA biosensor employing the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant enzyme (SEQ ID NO: 5) sampled at 5, 30, and 60 second timepoints.

[0349] The miniaturized needle or wire type L-DOPA biosensor was further tested by measuring an open circuit potential curves vs time after pulsing a chronoamperometry signal at 0.3V for 180 seconds (**Figure 19A**). The generated L-DOPA open circuit potential calibration curve was taken at 240 second after the voltage pulse and measured from 0.1-55 μM of L-DOPA, giving an RSD value of 0.99 (**Figure 19B**). The respective limit of detection (LOD) of L-DOPA was found by taking the $\text{STD} \times 3$, giving a value of 0.25 μM . The sensor was further tested and validated using human plasma as the sample, as shown in **Figure 19C** which shows open circuit potential measurements from 0-100 μM and the concentration gradient between 0-0.2 μM L-DOPA fit to a linear slope. The respective LOD was found to be 170 nM L-DOPA, further confirming the potential of this biosensing scheme for L-DOPA detection in Parkinson's patients.

[0350] In a preferred embodiment, an overpotential of 0.3 V, with 100 μM DSH was selected based on the optimization of signal sensitivity as compared to the impact of interferents on the measured signal (**Figures 40A-40C, and Figure 46**). The potential was applied for 180 seconds,

followed by measurement of OCP for 3 minutes, while also calculating the transient OCP response. This algorithm was then cycled over each additional concentration of levodopa giving three distinct sensor signals which correlate to L-DOPA (**Figures 42A-42C**). All three metrics clearly depict levodopa-dependent changes across both the cerebral spinal fluid and plasma therapeutic ranges. **Figures 32A-32C** display a chronoamperometric, open circuit potential, and transient open circuit potential calibration curve against levodopa, with all three Pearson Coefficients being greater than 0.93 across the entire therapeutic range (0-55 μM). The limits of detection for these three metrics are 138 nM, 72.8 nM, and 616 nM for chronoamperometric, OCP, and transient OCP, respectively. This clearly shows the efficacy of the sensor to measure clinically relevant changes in levodopa across therapeutic dosages. Standard error was calculated using 9 replicate needle or wire-type sensors.

[0351] The wire type sensor was also used in filtered human serum, evaluating levodopa from the range of 0 - 55 μM , using the electrochemical protocol described in **Figure 26A**. The results of this test are shown in **Figure 31A-31F**, displaying high correlation using chronoamperometry, open circuit potential, and transient open circuit potential across the entire therapeutic window. Additionally, transient open circuit potential curves were measured vs time after pulsing a chronoamperometry signal at 0.3V for 180 seconds (**Figure 20A**). The generated L-DOPA open circuit potential calibration curve was taken at 240 second after the voltage pulse and measured from 0.1-55 μM of L-DOPA (**Figure 20B**). The respective LOD of L-DOPA was found by taking the $\text{STD} \times 3$, giving a value of 0.22 μM . Further testing of the sensor was performed using human plasma as the sample, providing the transient open circuit potential measurements shown in **Figure 20C**.

[0352] Chronoamperometric curves were also measured vs time while holding the voltage at 0.3V vs the Ag/Cl reference electrode (**Figure 21A**). The generated L-DOPA chronoamperometric calibration curve was taken at 3 second after the voltage pulse and measured from 0.1-55 μM of L-DOPA (**Figure 21B**). The respective limit of detection LOD of L-DOPA was found by taking the $\text{STD} \times 3$, giving a value of 1.12 μM .

[0353] Additionally, the gold wire-type sensor with the engineered copper dehydrogenase was tested to measure L-DOPA in a flow cell setup. The observed raw data (**Figure 25A**, and **Figures 35A and 35C**) verifies the reversible characteristic of this biosensing approach. The sensor reliably detected L-DOPA in a concentration range of 0-55 μM (**Figure 25B and Figure**

35B and 35D), both when the concentration was increasing and decreasing, validating the sensitivity and performance of the L-DOPA sensor. Using a similar flow cell design, various needle or wire type sensors were evaluated with increasing and decreasing L-DOPA, ranging from 0 – 15 μM over several hours of continuous measurement using the protocol described in **Figure 26A**. L-DOPA values were calculated over time using the calibration curves derived from **Figure 32A-32C**. **Figure 36A and 36B** show time course of both absolute levodopa concentration, and rate of change in levodopa using continuous levodopa sensor with two peristaltic pumps, varying the concentration between 0 – 15 μM , collecting data every 3 minutes. **Figure 36C** displays a zoomed in section of both absolute and change in levodopa over time, demonstrating the feasibility of being able to “predict” changes in levodopa 7 - 12 minutes prior to either a peak or valley concentration. The curve displaying the real time change in levodopa, enables the ability to “predict” or intervene prior to the onset of symptoms.

[0354] The LODs for the L-DOPA biosensor measured through various techniques were well within physiological ranges, showing utility for L-DOPA detection for Parkinson’s disease patients. As indicated by this data, a miniaturized needle type L-DOPA biosensor was successfully realized, and its L-DOPA dependent signal was reliably measured and confirmed.

Example 6: The L-DOPA biosensor has minimal signal loss over extended storage stability

[0355] In this embodiment gold disk electrodes were polished using 0.3 and 0.05 μm aluminum powder followed by soaking in piranha (3:1 sulfuric acid to hydrogen peroxide) for 1 hour. After soaking, each electrode was electrochemically cleaned using cyclic voltammetry in 50 mM KOH solution, sweeping from 0 - -1.2 V. 100 μM DSH was then immobilized using acetone overnight on a shaker at 25°C at 300 rpm. Following this incubation, 0.014 mg/mL of the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant enzyme (SEQ ID NO: 5) was immobilized overnight at 25°C at 300 rpm, then stored in 100 mM potassium phosphate buffer until further testing. Electrodes were stored in batches of $n = 3$ for 1 – 7 days, as well as 7 – 21 days. On various days electrodes were removed from the buffer storage solution, and evaluated from 0 – 55 μM L-DOPA, displaying little to no drift in signal as a function of storage time (**Figures 34A and 34B, and 51A-51C**)

Example 7: Screen-printed, single use, disposable sensor strips for point-of-care testing and personal use L-DOPA monitoring

[0356] Gold screen-printed electrodes were rinsed with ethanol and distilled water, then the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant enzyme (SEQ ID NO: 5) was drop casted on the electrode surface at 0.1, 0.5, and 1 mg/mL concentrations. The biosensor strip was tested at a 5 μ M concentration of L-DOPA, providing open circuit potential measurements shown in **Figure 22A** and amperometric measurements shown in **Figure 22C**. The biosensor strip was also tested at a 20 μ M concentration of L-DOPA, providing open circuit potential measurements shown in **Figure 22B** and amperometric measurements shown in **Figure 22D**.

[0357] The results indicate that sensor strips employing the engineered copper dehydrogenases disclosed herein can detect L-DOPA at physiologically relevant concentrations. These single use, disposable sensor strips would be practical and highly useful for point-of-care testing (POCT) and for personal use. The L-DOPA monitoring system provided herein could improve treatment and quality of life for PD patients through facile and accurate detection of L-DOPA with engineered copper dehydrogenases.

Example 8: An Enzymatic biofuel cell with a copper dehydrogenase anode

[0358] Multicopper oxidases have been used as cathodes in enzyme fuel cells, involved in reducing ambient oxygen into water. The engineered copper dehydrogenases disclosed herein have reduced oxidase function and can act as anodic enzyme catalysts. An enzyme fuel cell was fabricated with a copper dehydrogenase (SEQ ID NO: 5) immobilized on the anode, and a PaMCO (SEQ ID NO: 7) immobilized on the cathode.

[0359] Anodes and cathodes were prepared by first cleaning Toray Carbon Paper electrodes by spraying with deionized water. Next, Ketjenblack Ink was prepared by mixing Ketjenblack powder with triton and deionized water, and then dispersed via ultrasonication. To prepare the anode, a mixture of Ketjenblack ink, the engineered PaMCO Phe290Ile/His396Ala/His459Ala mutant enzyme (SEQ ID NO: 5), and PPB was prepared and spotted onto the Toray Carbon Paper electrode. To prepare the cathode, a mixture of PaMCO Phe290Ile enzyme (SEQ ID NO: 7) and PPB was prepared and spotted onto the Toray Carbon Paper electrode. In one optimized protocol, 4 μ L of Ketjenblack was used with 20 μ L of enzyme (e.g., a 1:5 ratio of Ketjenblack ink to enzyme) and 36 μ L of PPB. Ratios of Ketjenblack ink to

enzyme of 1:1 to 1:20 were tested. The electrodes were dried and cross-linked by vapor deposition of glutaraldehyde. Afterwards, the electrodes were dipped in Tris-HCl and stored in PPB until use.

[0360] Electrodes were tested in an electrochemical cell containing 100 mM PPB. Both electrodes were placed in the electrochemical cell, connected to a potentiostat, and the open circuit potential was measured as different resistance was applied.

[0361] As shown in the power output (**Figure 24A**) and current density (**Figure 24B**) measurements, the engineered copper dehydrogenase successfully catalyzed an anodic reaction to power an enzyme fuel cell. Thus, engineered copper dehydrogenases can power enzyme fuel cells, which have potential for use in non-toxic and biocompatible batteries for biomedical devices.

[0362] Many modifications and other embodiments of the inventions set forth herein will come to mind to one skilled in the art to which the inventions pertain having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the inventions are not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

CLAIMS

What is claimed is:

1. A copper dehydrogenase engineered from a multicopper oxidase to have reduced oxidase activity as compared to a wild-type multicopper oxidase.
2. The copper dehydrogenase of claim 1, wherein the oxidase activity is reduced by at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% as compared to the oxidase activity of the wild-type multicopper oxidase.
3. The copper dehydrogenase of claim 1 or 2, wherein the reduced oxidase activity results from modification at one or more amino acid residues, wherein the one or more amino acid residues are one or more type 2 (T2) and/or type 3 (T3) copper atom ligands.
4. The copper dehydrogenase of claim 3, wherein the T2 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 64 and 366 of SEQ ID NO: 7, and wherein the T3 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 66, 104, 106, 368, 431, and 433 of SEQ ID NO: 7.
5. The copper dehydrogenase of claim 3, wherein:
 - (i) the T2 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 101 and 446 of SEQ ID NO: 19, and wherein the T3 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 103, 141, 143, 448, 498 and 500 of SEQ ID NO: 19;
 - (ii) the T2 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 105 and 422 of SEQ ID NO: 20, and the T3 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 107, 153, 155, 424, 491 and 493 of SEQ ID NO: 20;
 - (iii) the T2 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 95 and 400 of SEQ ID NO: 21, and the T3 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 97, 135, 137, 402, 449 and 451 of SEQ ID NO: 21;

(iv) the T2 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 132 and 439 of SEQ ID NO: 22, and the T3 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 134, 171, 173, 441, 494 and 451 of SEQ ID NO: 22;

(v) the T2 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 103 and 422 of SEQ ID NO: 23, and the T3 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 105, 151, 153, 424, 491 and 493 of SEQ ID NO: 23;

(vi) the T2 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 103 and 421 of SEQ ID NO: 24, and the T3 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 151, 153, 423, 490 and 492 of SEQ ID NO: 24;

(vii) the T2 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 145 and 511 of SEQ ID NO: 25, and the T3 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 147, 185, 187, 513, 586 and 588 of SEQ ID NO: 25; or

(viii) the T2 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 145 and 514 of SEQ ID NO: 26, and the T3 ligand is an amino acid residue corresponding to a position selected from the group consisting of positions 147, 185, 187, 516, 589 and 591 of SEQ ID NO: 26.

6. The copper dehydrogenase of any one of claims 1-5, with increased dehydrogenase activity towards a substrate as compared to the wild-type multicopper oxidase.

7. The copper dehydrogenase of claim 6, wherein the dehydrogenase activity of the copper dehydrogenase is increased by at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 100% as compared to the dehydrogenase activity of the wild-type multicopper oxidase.

8. The copper dehydrogenase of claim 6 or 7, wherein the substrate is a phenolic compound.

9. The copper dehydrogenase of claim 8, wherein the substrate is L-DOPA.
10. The copper dehydrogenase of claim 8, wherein the substrate is a phenolic compound derived from lignin, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 2,6-dimethoxyphenol, 4-methoxyphenol, phenol, hydroquinone, 2,4-dichlorophenol, p-benzoquinone, o-benzenediol, m-benzenediol, or p-benzenediol.
11. The copper dehydrogenase of any one of claims 1-9, comprising a sequence having at least 90% sequence identity to any one of SEQ ID NOs:1-6, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 64, 66, 104, 106, 366, 368, 431, and 433 of SEQ ID NO: 7 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 7.
12. The copper dehydrogenase of claim 11, comprising a modification of one or more amino acid residues at a position selected from:
- (a) a position corresponding to position 66 of the amino acid sequence set forth in SEQ ID NO:7;
 - (b) a position corresponding to position 104 of the amino acid sequence set forth in SEQ ID NO:7;
 - (c) a position corresponding to position 368 of the amino acid sequence set forth in SEQ ID NO:7; and
 - (d) a position corresponding to position 431 of the amino acid sequence set forth in SEQ ID NO:7.
13. The copper dehydrogenase of any one of claims 1-10, comprising:
- (i) a sequence having at least 90% sequence identity to SEQ ID NO: 19, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 101, 103, 141, 143, 446, 448, 498 and 500 of SEQ ID NO: 19 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 19;
 - (ii) a sequence having at least 90% sequence identity to SEQ ID NO: 20, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 105, 107, 153, 155, 422, 424, 491 and 493 of SEQ ID NO:

20 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 20;

(iii) a sequence having at least 90% sequence identity to SEQ ID NO: 21, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 95, 97, 135, 137, 400, 402, 449 and 451 of SEQ ID NO: 21 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 21;

(iv) a sequence having at least 90% sequence identity to SEQ ID NO: 22, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 132, 134, 171, 173, 439, 441, 494 and 496 of SEQ ID NO: 22 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 22;

(v) a sequence having at least 90% sequence identity to SEQ ID NO: 23, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 103, 105, 151, 153, 422, 424, 491 and 493 of SEQ ID NO: 23 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 23;

(vi) a sequence having at least 90% sequence identity to SEQ ID NO: 24, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 103, 105, 151, 153, 421, 423, 490 and 492 of SEQ ID NO: 24 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 24;

(vii) a sequence having at least 90% sequence identity to SEQ ID NO: 25, provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 145, 147, 185, 187, 511, 513, 586 and 588 of SEQ ID NO: 25 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 25; or

(viii) a sequence having at least 90% sequence identity to SEQ ID NO: 26 provided that at least one of the amino acids at a position in said sequence corresponding to a position selected from the group consisting of positions 145, 147, 185, 187, 514, 516, 589 and 591 of SEQ ID NO:

26 is different from the amino acid occupying the corresponding position according to SEQ ID NO: 26.

14. The copper dehydrogenase of claim 13, comprising:

(i) a modification of one or more histidine residues selected from: (a) a position corresponding to position 103 of the amino acid sequence set forth in SEQ ID NO: 19; (b) a position corresponding to position 141 of the amino acid sequence set forth in SEQ ID NO: 19; (c) a position corresponding to position 448 of the amino acid sequence set forth in SEQ ID NO: 19; and (d) a position corresponding to position 498 of the amino acid sequence set forth in SEQ ID NO: 19;

(ii) a modification of one or more histidine residues selected from: (a) a position corresponding to position 107 of the amino acid sequence set forth in SEQ ID NO: 20; (b) a position corresponding to position 153 of the amino acid sequence set forth in SEQ ID NO: 20; (c) a position corresponding to position 424 of the amino acid sequence set forth in SEQ ID NO: 20; and (d) a position corresponding to position 491 of the amino acid sequence set forth in SEQ ID NO: 20;

(iii) a modification of one or more histidine residues selected from: (a) a position corresponding to position 97 of the amino acid sequence set forth in SEQ ID NO: 21; (b) a position corresponding to position 135 of the amino acid sequence set forth in SEQ ID NO: 21; (c) a position corresponding to position 402 of the amino acid sequence set forth in SEQ ID NO: 21; and (d) a position corresponding to position 449 of the amino acid sequence set forth in SEQ ID NO: 21;

(iv) a modification of one or more histidine residues selected from: (a) a position corresponding to position 134 of the amino acid sequence set forth in SEQ ID NO: 22; (b) a position corresponding to position 171 of the amino acid sequence set forth in SEQ ID NO: 22; (c) a position corresponding to position 441 of the amino acid sequence set forth in SEQ ID NO: 22; and (d) a position corresponding to position 494 of the amino acid sequence set forth in SEQ ID NO: 22;

(v) a modification of one or more histidine residues selected from: (a) a position corresponding to position 105 of the amino acid sequence set forth in SEQ ID NO: 23; (b) a position corresponding to position 151 of the amino acid sequence set forth in SEQ ID NO: 23; (c) a position corresponding to position 424 of the amino acid sequence set forth in SEQ ID NO: 23;

and (d) a position corresponding to position 491 of the amino acid sequence set forth in SEQ ID NO: 23;

(vi) a modification of one or more histidine residues selected from: (a) a position corresponding to position 105 of the amino acid sequence set forth in SEQ ID NO: 24; (b) a position corresponding to position 151 of the amino acid sequence set forth in SEQ ID NO: 24; (c) a position corresponding to position 423 of the amino acid sequence set forth in SEQ ID NO: X6; and (d) a position corresponding to position 490 of the amino acid sequence set forth in SEQ ID NO: 24;

(vii) a modification of one or more histidine residues selected from: (a) a position corresponding to position 147 of the amino acid sequence set forth in SEQ ID NO: 25; (b) a position corresponding to position 185 of the amino acid sequence set forth in SEQ ID NO: 25; (c) a position corresponding to position 513 of the amino acid sequence set forth in SEQ ID NO: 25; and (d) a position corresponding to position 586 of the amino acid sequence set forth in SEQ ID NO: 25; or

(viii) a modification of one or more histidine residues selected from: (a) a position corresponding to position 147 of the amino acid sequence set forth in SEQ ID NO: 26; (b) a position corresponding to position 185 of the amino acid sequence set forth in SEQ ID NO: 26; (c) a position corresponding to position 516 of the amino acid sequence set forth in SEQ ID NO: 26; and (d) a position corresponding to position 589 of the amino acid sequence set forth in SEQ ID NO: 26.

15. The copper dehydrogenase of claim 12 or 14, wherein the modification is a substitution with a different amino acid residue.

16. The copper dehydrogenase of claim 15, wherein the modification is a substitution of a histidine residue with an alanine residue.

17. The copper dehydrogenase of claim 12, comprising a modification at one or more amino acid residues at a position selected from:

(a) a position corresponding to position 66 of the amino acid sequence set forth in SEQ ID NO: 7, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue;

(b) a position corresponding to position 104 of the amino acid sequence set forth in SEQ ID NO:7, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue;

(c) a position corresponding to position 368 of the amino acid sequence set forth in SEQ ID NO:7, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; and

(d) a position corresponding to position 431 of the amino acid sequence set forth in SEQ ID NO:7, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue.

18. The copper dehydrogenase of claim 12, comprising a modification at one or more amino acid residues at a position selected from:

(a) a position corresponding to position 368 of the amino acid sequence set forth in SEQ ID NO:7, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue; and

(b) a position corresponding to position 431 of the amino acid sequence set forth in SEQ ID NO:7, wherein the modification includes a substitution of the wild-type amino acid residue with an alanine residue.

19. The copper dehydrogenase of any one of claims 4, 11, 12, 17, and 18, comprising a substitution of the wild-type amino acid residue corresponding to position 262 of the amino acid sequence set forth in SEQ ID NO: 7.

20. The copper dehydrogenase of claim 19, wherein the wild-type amino acid residue corresponding to position 262 of the amino acid sequence set forth in SEQ ID NO: 7 is a phenylalanine residue, and the substitution comprises changing the wild-type phenylalanine residue to an isoleucine residue.

21. The copper dehydrogenase of any one of claims 11, 12, and 15-20, having at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 99.9% sequence identity to any one of SEQ ID NOs:1-6.

22. The copper dehydrogenase of claim 21, comprising any one of the amino acid sequences set forth in SEQ ID NOs:1-6.

23. A method of assaying L-DOPA in a sample, the method comprising the steps of: contacting the sample with the copper dehydrogenase of any one of claims 1-22; and measuring an amount of L-DOPA.

24. The method of claim 23, wherein the measurement is an open-circuit potential, chronoamperometry, square-wave voltammetry, or extended gate field effect transistor (EGFET) measurement.

25. The method of claim 24, wherein the measurement is a transient open circuit potential measurement.

26. The method of claim 24 or 25, wherein the method comprises measuring a combination of two or more of open-circuit potential, transient open circuit potential, chronoamperometry, square-wave voltammetry, or extended gate field effect transistor (EGFET) measurements.

27. The method of any one of claims 23-26, wherein the measurement is a continuous measurement.

28. The method of any one of claims 23-26, wherein the measurement is an intermittent measurement.

29. The method of claim 28, wherein the intermittent measurement is taken at an interval of 30 seconds to 10 minutes.

30. The method of any one of claims 23-29, wherein the L-DOPA is derived from foslevodopa, and the method further comprises contacting the sample with an enzyme capable of dephosphorylating foslevodopa to L-DOPA before contacting the sample with the copper dehydrogenase.

31. A device for assaying L-DOPA in a sample, the device comprising the copper dehydrogenase of any one of claims 1-22.

32. The device of claim 31, further comprising:
- (I) a three-electrode configuration comprising a working electrode, a reference electrode, and a counter electrode; or
 - (II) a two-electrode configuration comprising a working electrode and a shared reference and a counter electrode.
33. The device of claim 31 or 32, wherein one or more electrodes are disk electrodes or needle electrodes.
34. The device of claim 31 or 32, wherein one or more electrodes are wire electrodes.
35. The device of any one of claims 31-34, wherein one or more electrodes are gold electrodes.
36. The device of any one of claims 31-35, wherein one or more electrodes are carbon, platinum or stainless-steel electrodes.
37. The device of any one of claims 31-36, wherein the enzyme is immobilized on the electrode by a self-assembled monolayer, a conductive hydrogel, entrapment, cross-linking, or physical adsorption.
38. The device of any one of claims 31-37, further comprising a sensor strip.
39. The device of claim 38, wherein the sensor strip is screen-printed.
40. The device of claim 38 or 39, wherein the sensor strip is single-use and/or disposable.
41. The device of any one of claims 31-40, wherein the device further comprises an enzyme capable of dephosphorylating foslevodopa to L-DOPA.
42. The device of claim 41, wherein the enzyme capable of dephosphorylating foslevodopa is a phosphatase.
43. A kit for assaying L-DOPA in a sample, the kit comprising the copper dehydrogenase of any one of claims 1-22.

44. An enzyme electrode comprising the copper dehydrogenase of any one of claims 1-22 immobilized on an electrode.

45. The enzyme sensor for assaying L-DOPA comprising the enzyme electrode of claim 44.

46. A polynucleotide encoding a copper dehydrogenase, said copper dehydrogenase comprising a modification of one or more amino acid residues at a position selected from: i) a position corresponding to position 66 of the amino acid sequence set forth in SEQ ID NO:7, ii) a position corresponding to position 104 of the amino acid sequence set forth in SEQ ID NO:7, iii) a position corresponding to position 368 of the amino acid sequence set forth in SEQ ID NO:7, and iv) a position corresponding to position 431 of the amino acid sequence set forth in SEQ ID NO:7.

47. A polynucleotide encoding the copper dehydrogenase of any one of claims 1-22.

48. The polynucleotide of claim 44, comprising a sequence set forth in any one of SEQ ID NOs: 11-16.

49. A method of producing the copper dehydrogenase of any one of claims 1-22, the method comprising the steps of:

- i) culturing a host cell transfected with a vector comprising a nucleic acid sequence encoding the copper dehydrogenase under conditions suitable for expression of the polypeptide from the host cell, and
- ii) recovering the copper dehydrogenase.

50. An anode comprising an electroconductive material, wherein the copper dehydrogenase of any one of claims 1-22 is immobilized on the anode and a substrate immobilized on the anode, wherein the substrate can be oxidized by the enzyme.

51. The anode of claim 50, wherein the substrate is formed by depositing a cross-linking reagent.

52. The anode of claim 51, wherein the cross-linking reagent is glutaraldehyde.

53. The anode of any one of claims 50-52, wherein the substrate is a phenolic compound.
54. The anode of claim 53, wherein the phenolic compound is derived from lignin.
55. The anode of any one of claims 51-54, wherein the electroconductive material is selected from the group consisting of carbon paper, glassy carbon, carbon nanotubes, gold, and palladium.
56. A fuel cell comprising the anode of any one of claims 50-55 and a cathode, wherein the cathode comprises an electroconductive material and an electron acceptor, wherein the anode and the cathode are in a solution, wherein the cathode is contact with dissolved oxygen.
57. The fuel cell of claim 56, wherein the electron acceptor of the cathode comprises a phenazine compound.
58. The fuel cell of claim 57, wherein the phenazine compound comprises phenazine ethosulfate (PES) or phenazine methosulfate (PMS).
59. The fuel cell of claim 57 or 58, wherein the phenazine compound is amine-reactive.
60. The fuel cell of claim 59, wherein the amine-reactive phenazine compound is N-hydroxysuccinimidylester 1-propoxy-5-ethylphenazinium ethyl sulfate (arPES).
61. The fuel cell of claim 56, wherein the electron acceptor of the cathode comprises a metal complex.
62. The fuel cell of claim 61, wherein the metal complex is an osmium complex or a ruthenium complex.
63. The fuel cell of any one of claims 56-62, wherein the electron acceptor of the cathode is immobilized on a protein.

64. The fuel cell of claim 63, wherein the protein is selected from the group consisting of bovine serum albumin (BSA), glucose dehydrogenase (GDH), lactate dehydrogenase (LDH), glucose oxidase (GOx), and lactate oxidase (LOx).

65. The fuel cell of claim 56, wherein the electron acceptor of the cathode is an oxidase enzyme.

66. The fuel cell of claim 65, wherein the oxidase enzyme of the cathode is a multicopper oxidase.

67. The fuel cell of claim 66, wherein the multicopper oxidase of the cathode is selected from the group laccase, bilirubin oxidase, ascorbic acid oxidase, and multicopper oxidase derived from *Pyrobaculum aerophilum*.

68. The fuel cell of claim 67, wherein the multicopper oxidase of the cathode is laccase derived from *Trametes versicolor* or *Aspergillus sp.*

69. The fuel cell of claim 67, wherein the multicopper oxidase of the cathode is bilirubin oxidase derived from *Myrothecium verrucaria*.

70. The fuel cell of any one of claims 56-69, wherein the solution of the anode and the solution of the cathode are in the same environment.

71. The fuel cell of any one of claims 56-69, wherein the solution of the anode and the solution of the cathode are in separate environments and connected by a salt bridge.

72. A battery comprising the fuel cell of any one of claims 56-71 and a container.

73. The battery of claim 72, comprising more than one fuel cell.

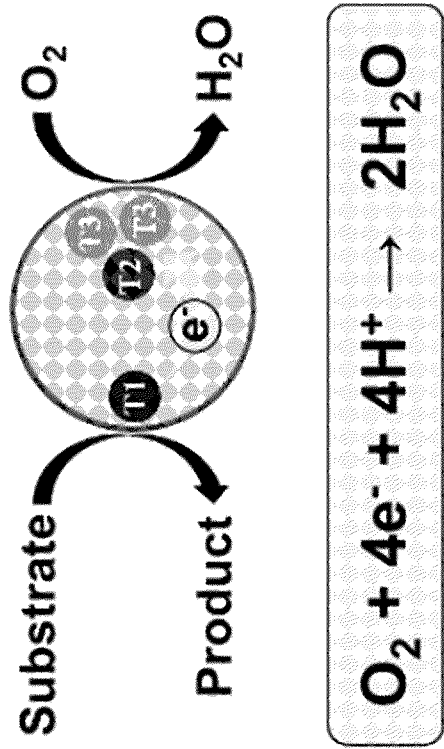
74. The battery of claim 73, wherein the fuel cells are in a series configuration.

75. The battery of claim 73, wherein the fuel cells are in a parallel configuration.

76. The battery of any one of claims 73-75, wherein the fuel cells are stacked.

77. The battery of any one of claims 72-76, comprising a biodegradable material.

Multicopper oxidase (MCO)



Characteristics

- Four copper atoms
- Direct four-electron transfer
- Production of water

Fig. 1

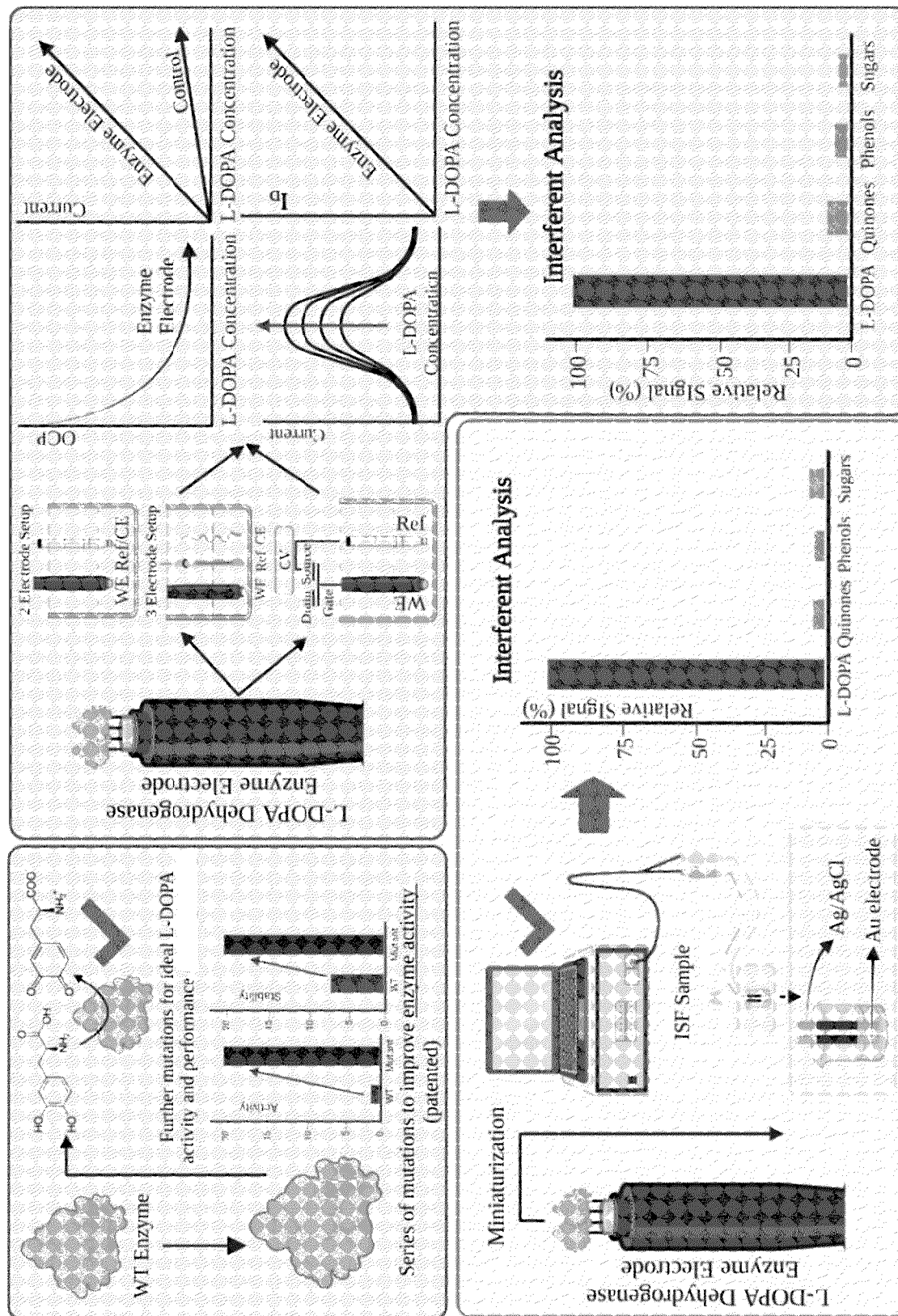
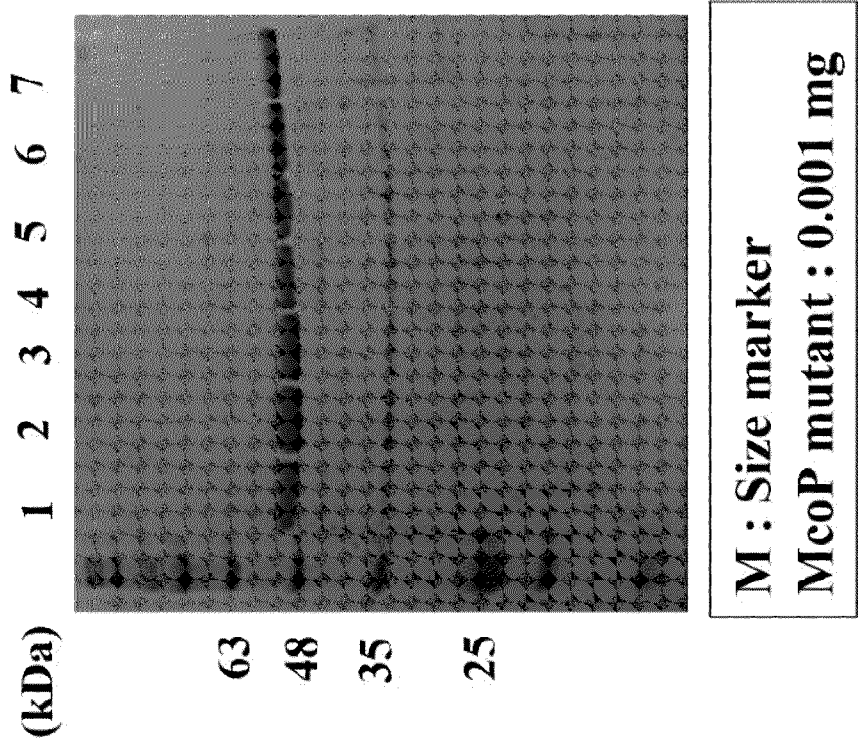


Fig. 2



Mutation	
SEQ ID NO: 1	H94A, F290I
SEQ ID NO: 2	H132A, F290I
SEQ ID NO: 3	H396A, F290I
SEQ ID NO: 4	H459A, F290I
SEQ ID NO: 5	H396A, H459A, F290I
SEQ ID NO: 6	H94A, H396A, H459A, F290I
SEQ ID NO: 7	F290I

Fig. 3

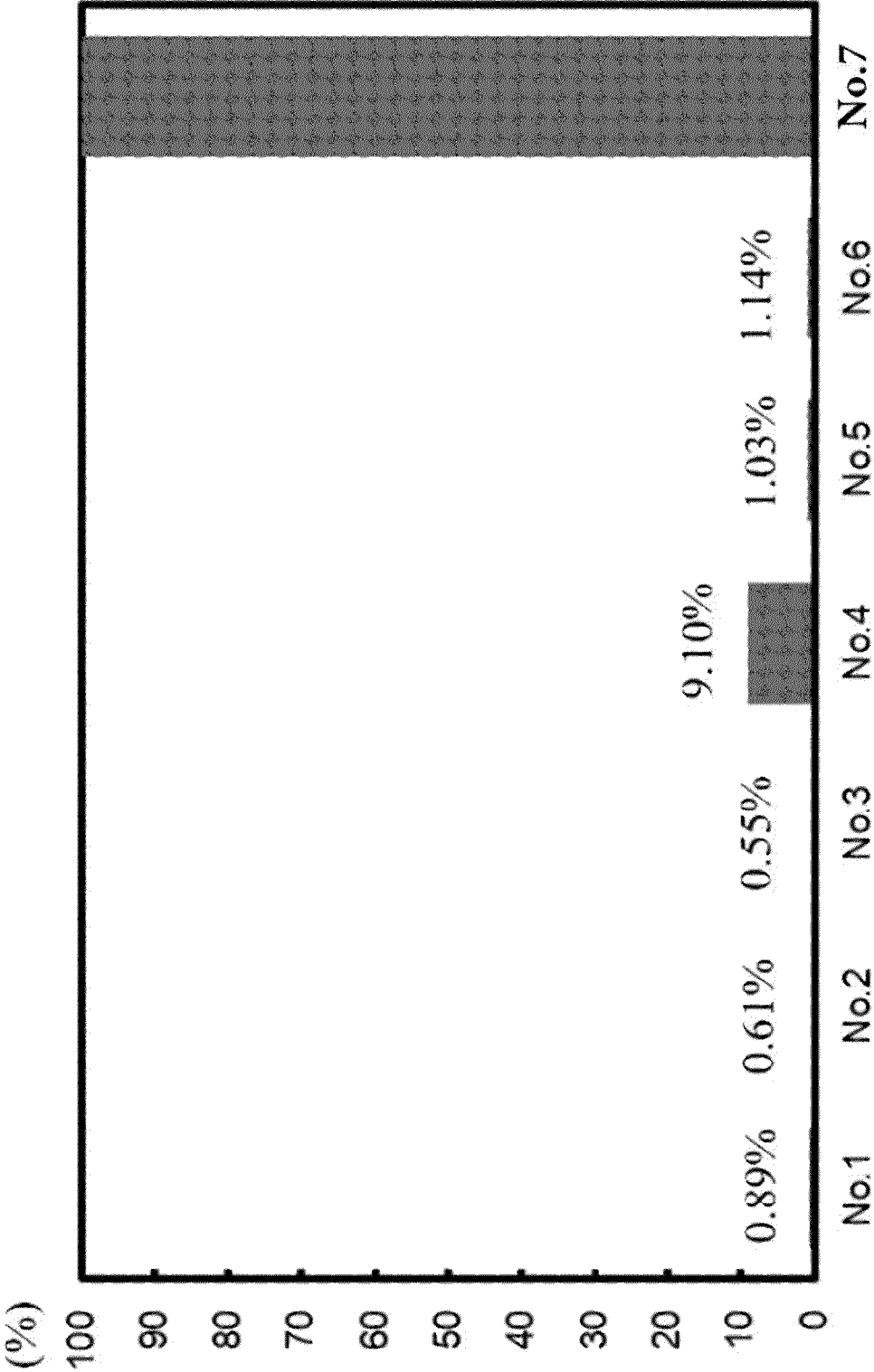


Fig. 4

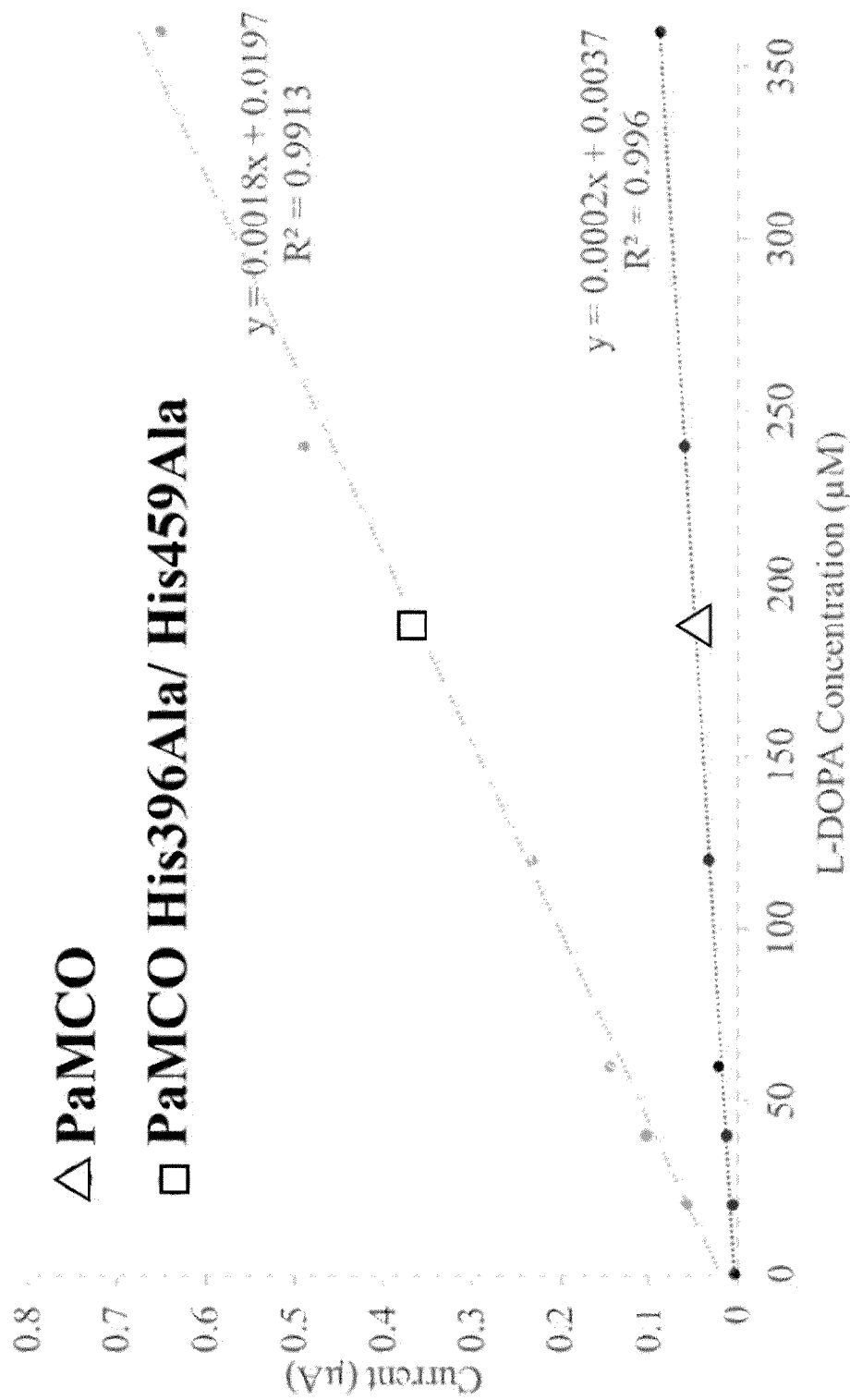


Fig. 5

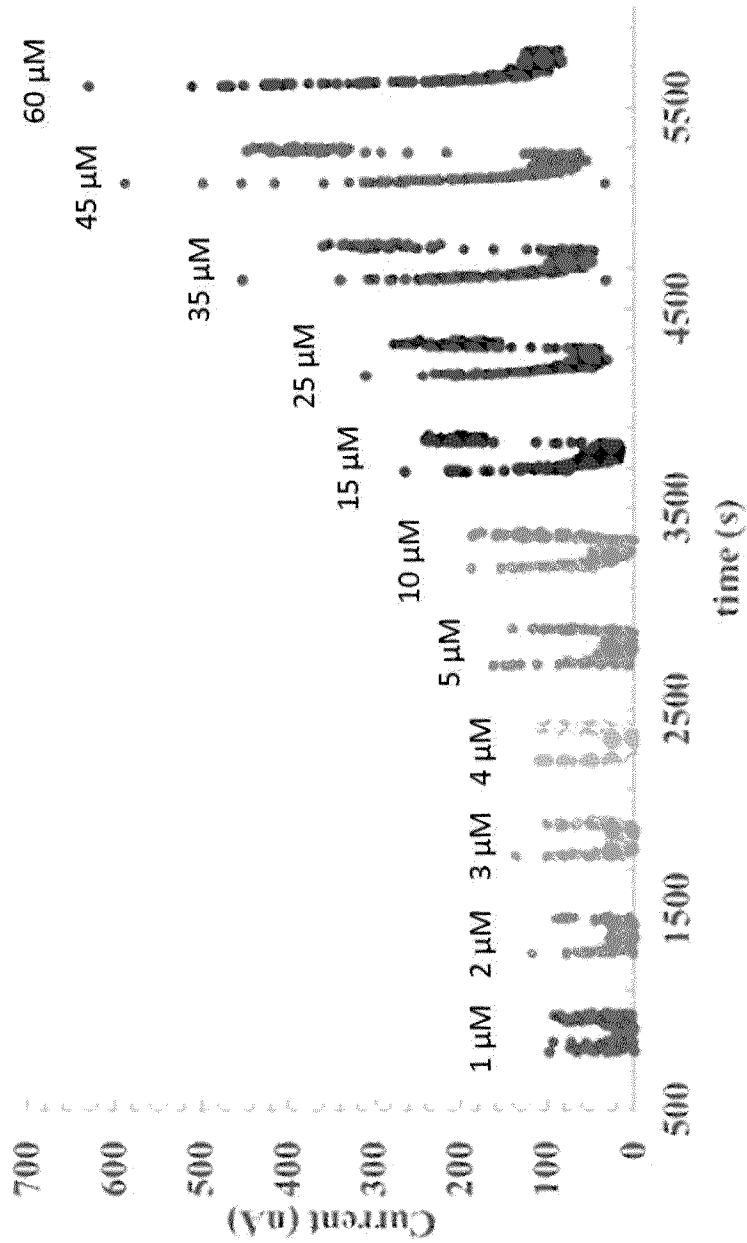


Fig. 6A

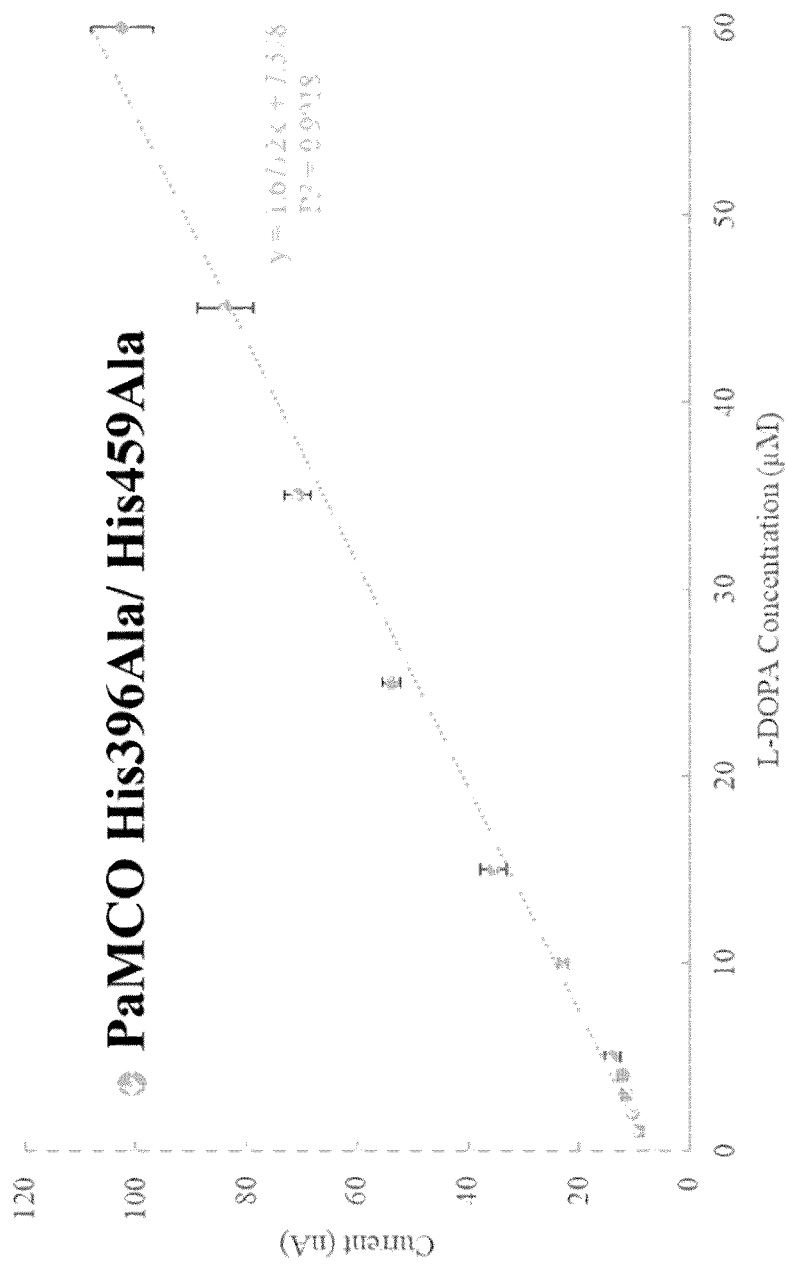


Fig. 6B

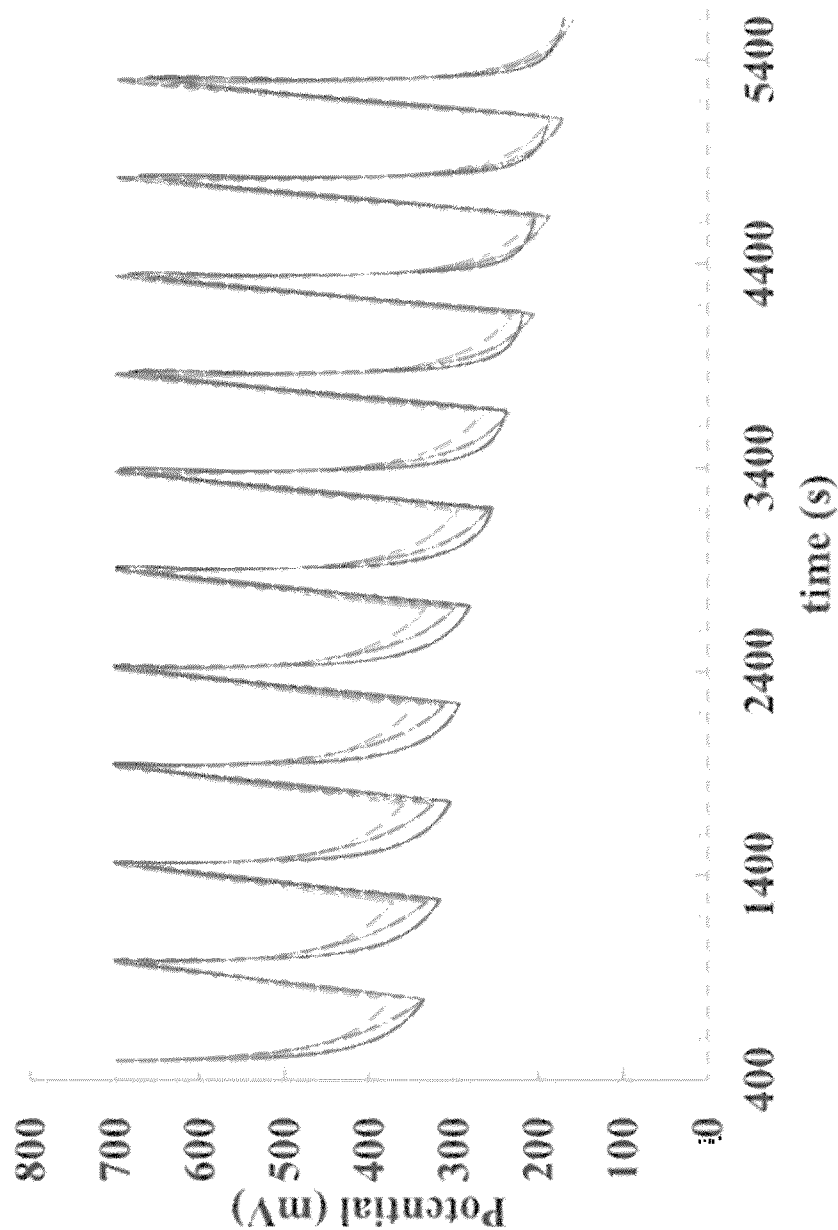


Fig. 7A

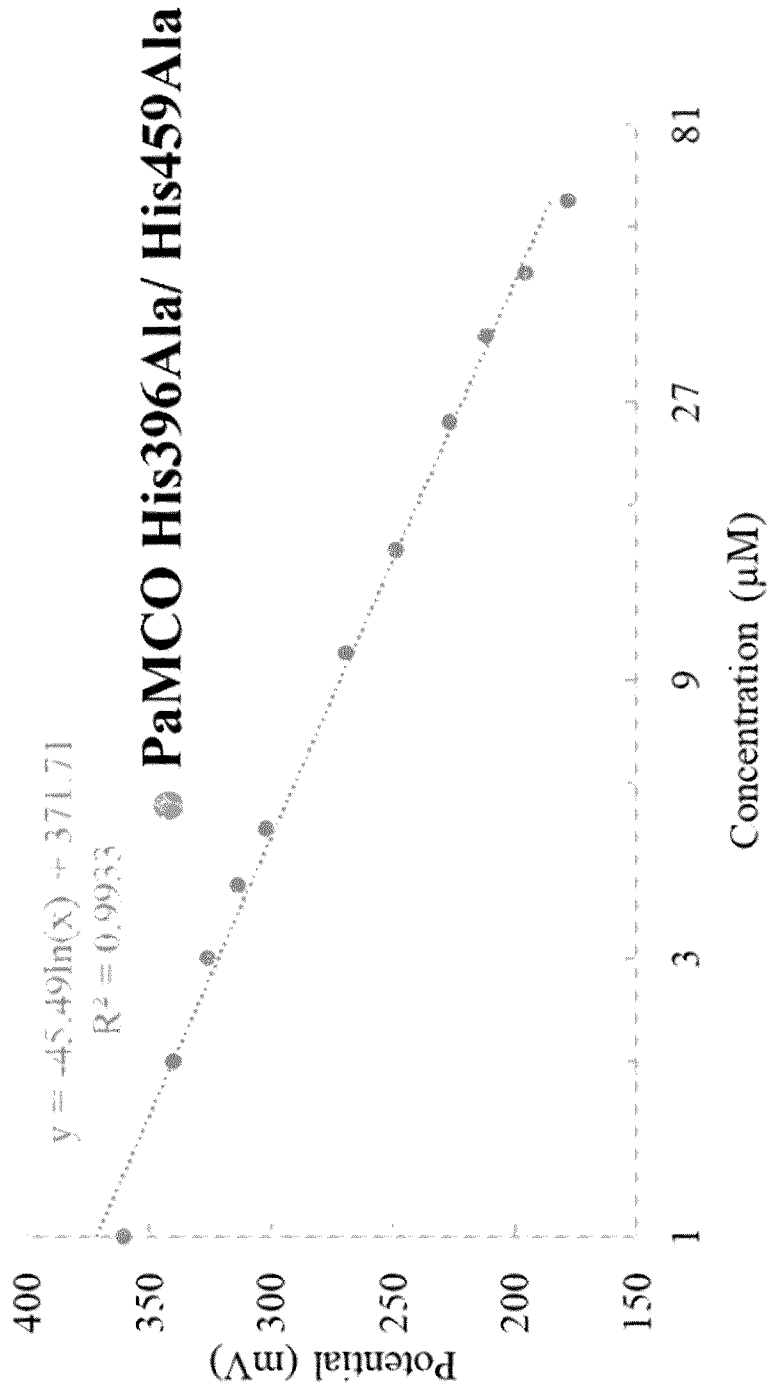


Fig. 7B

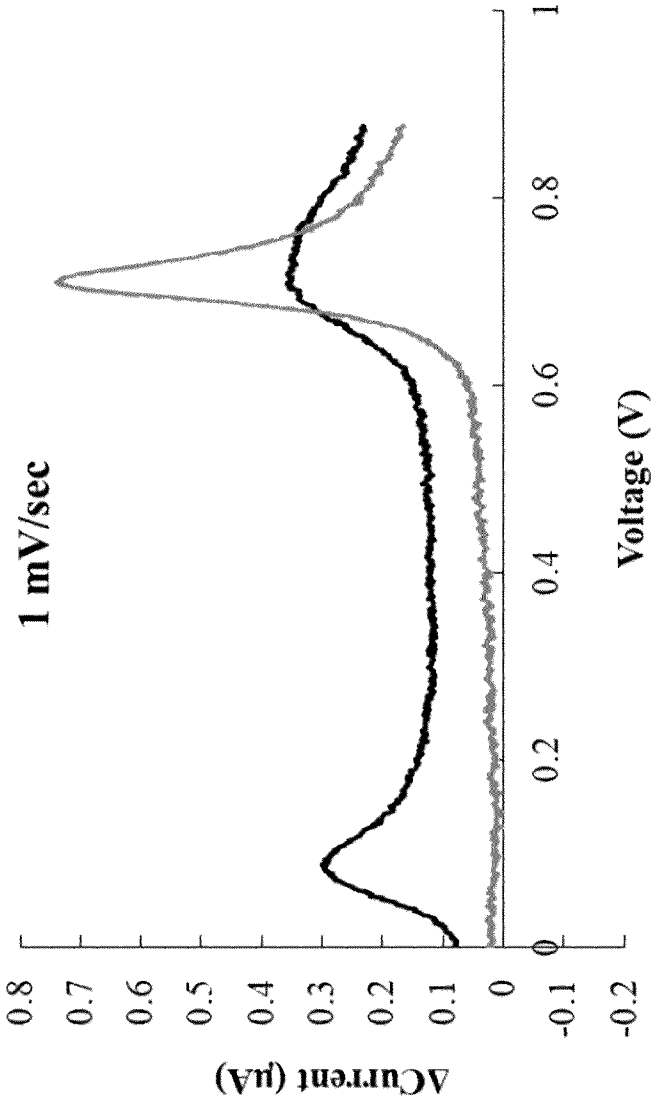


Fig. 8A

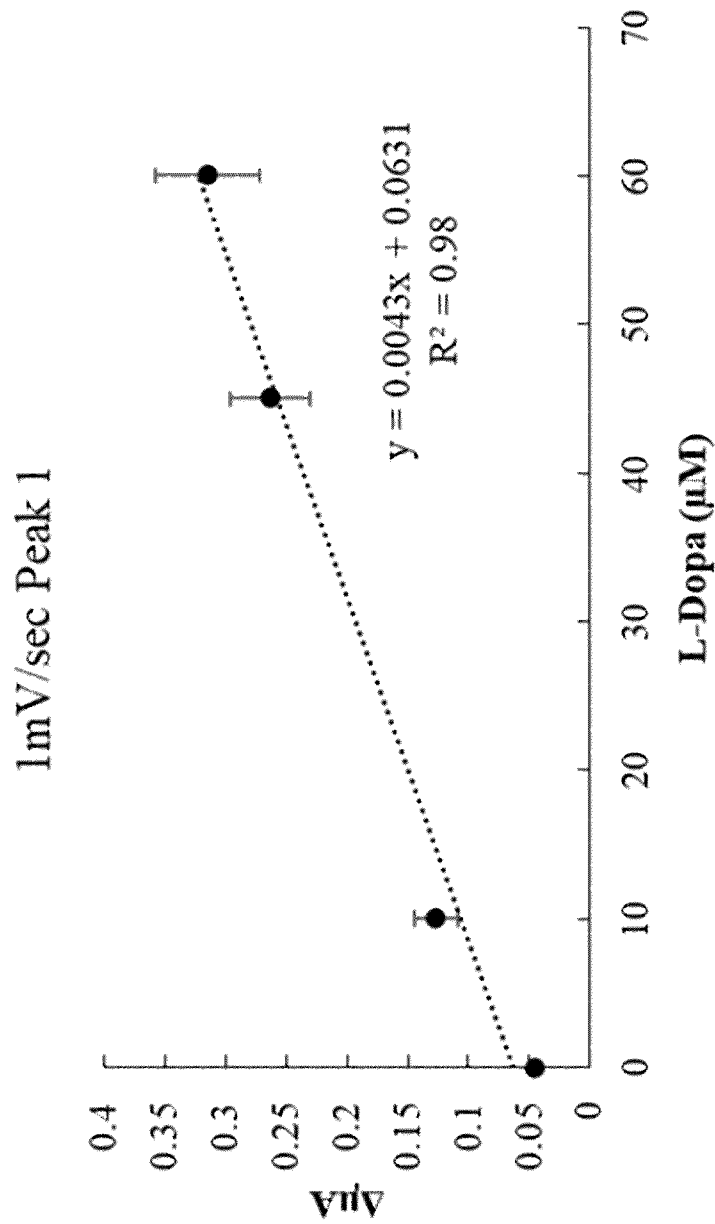


Fig. 8B

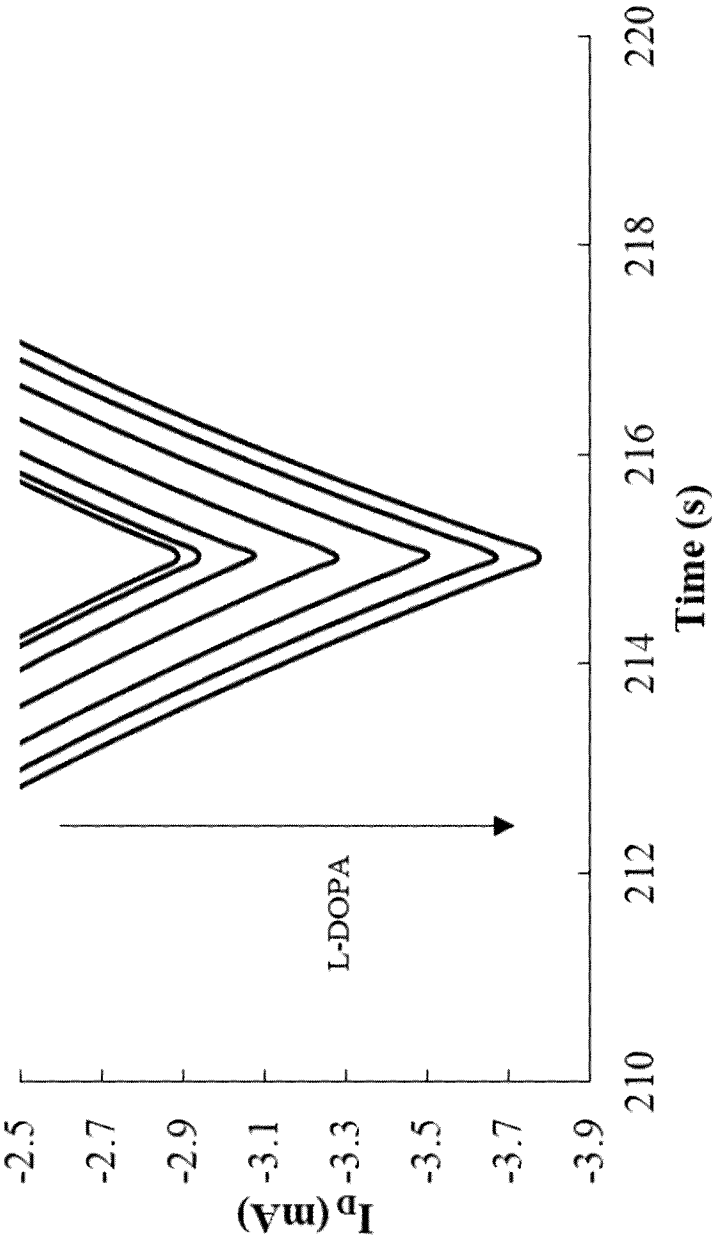


Fig. 9A

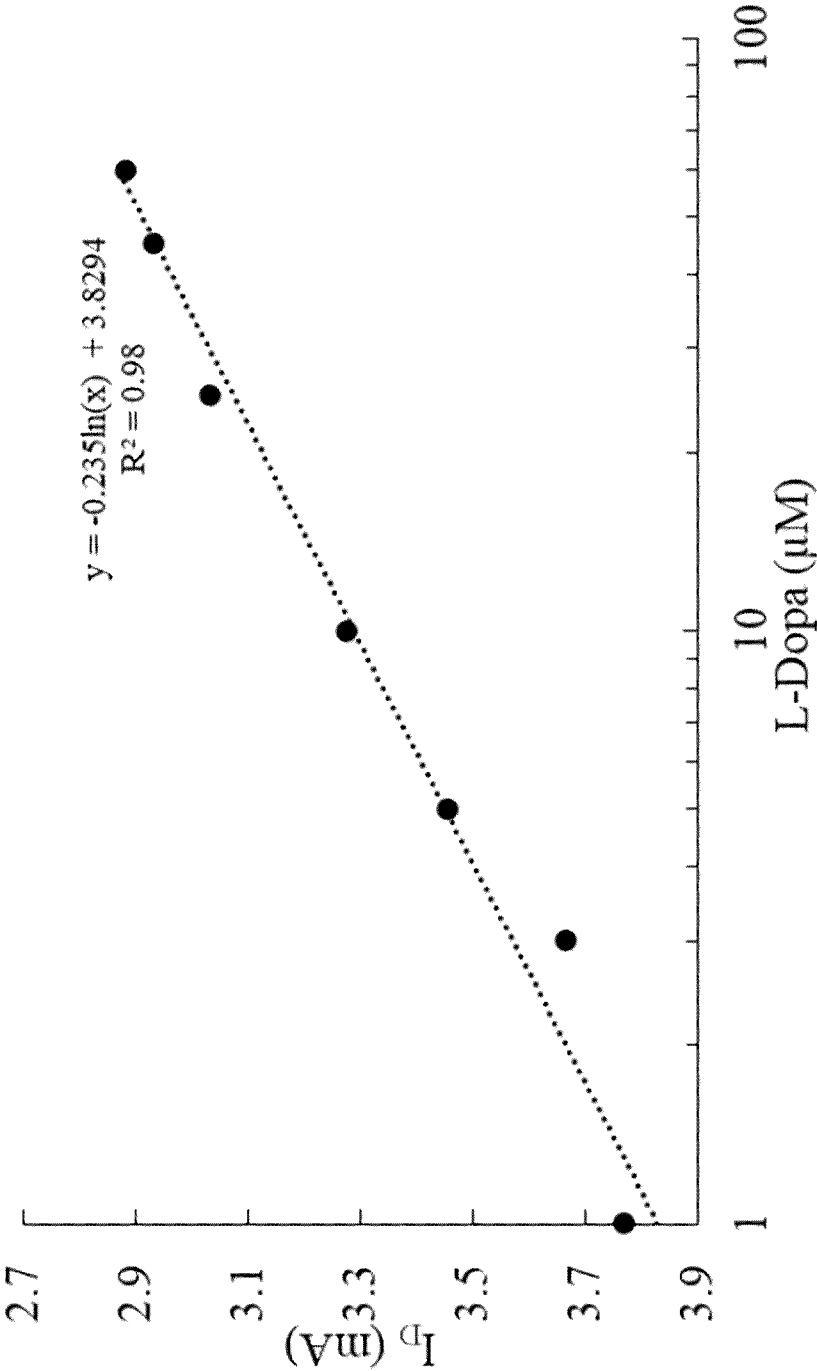


Fig. 9B

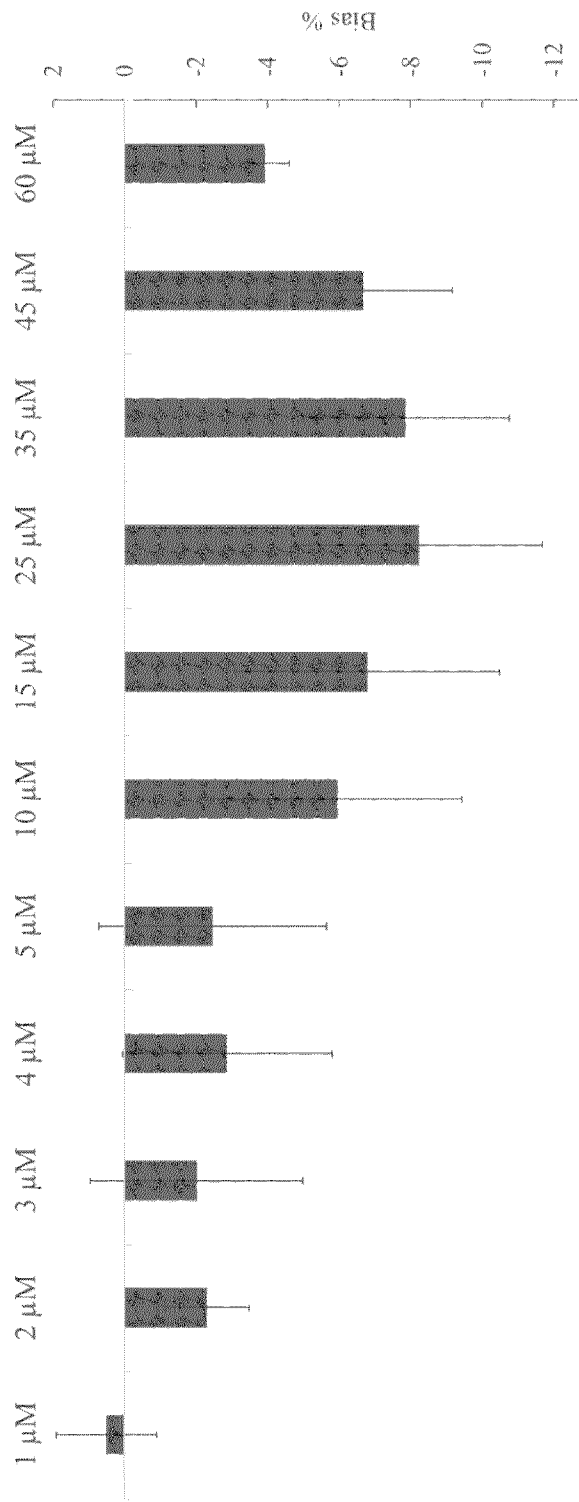


Fig. 10

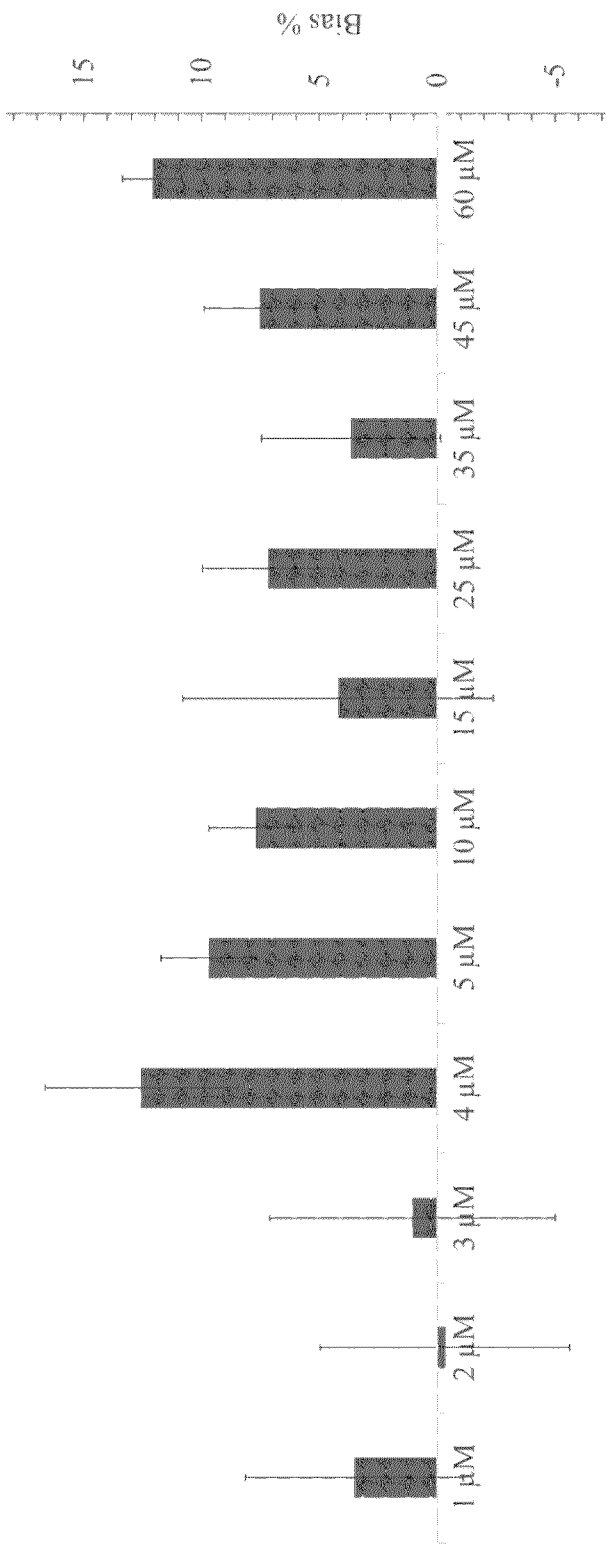
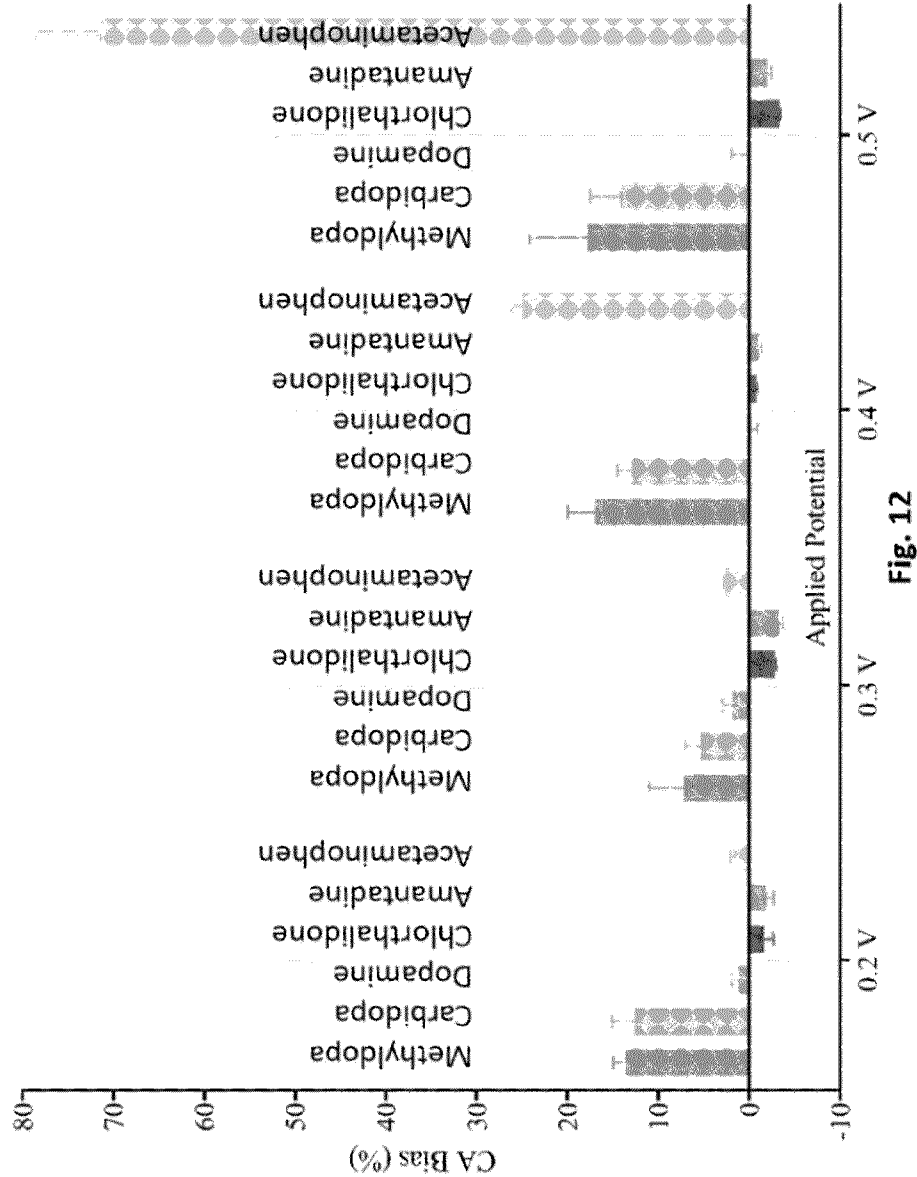


Fig. 11



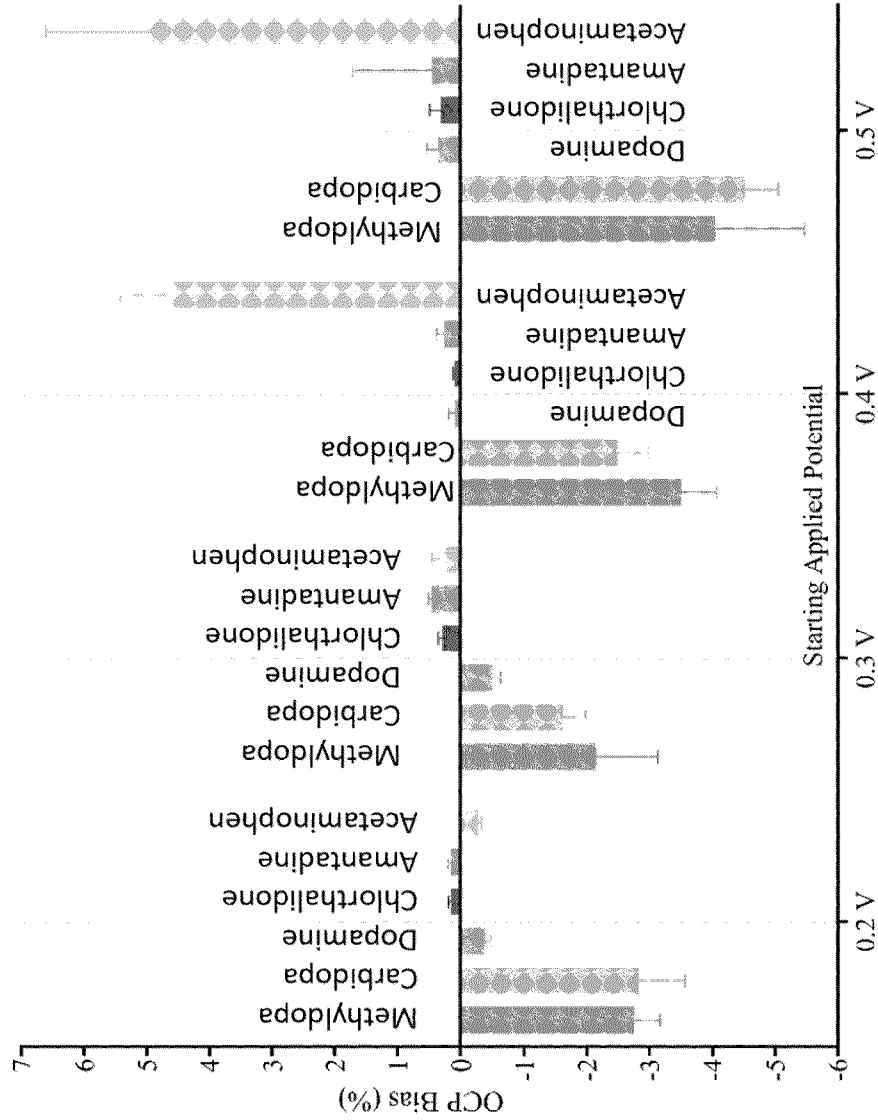


Fig. 13

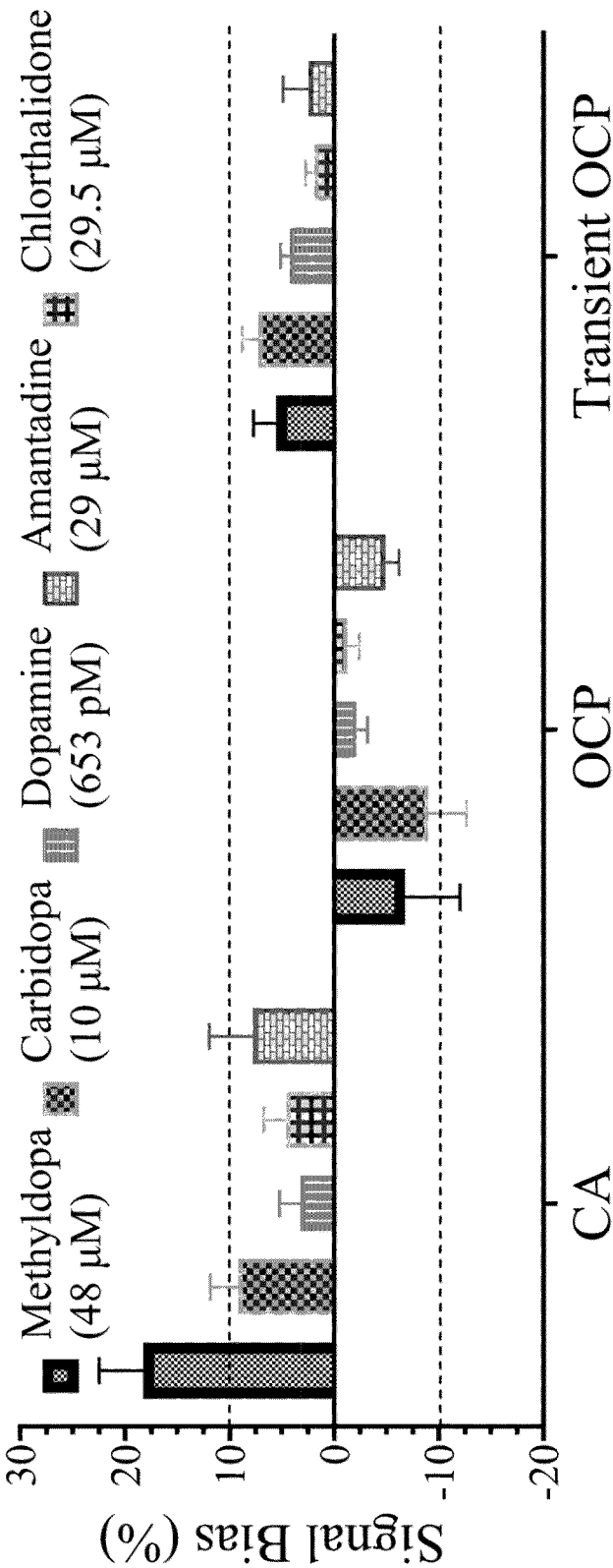


Fig. 14

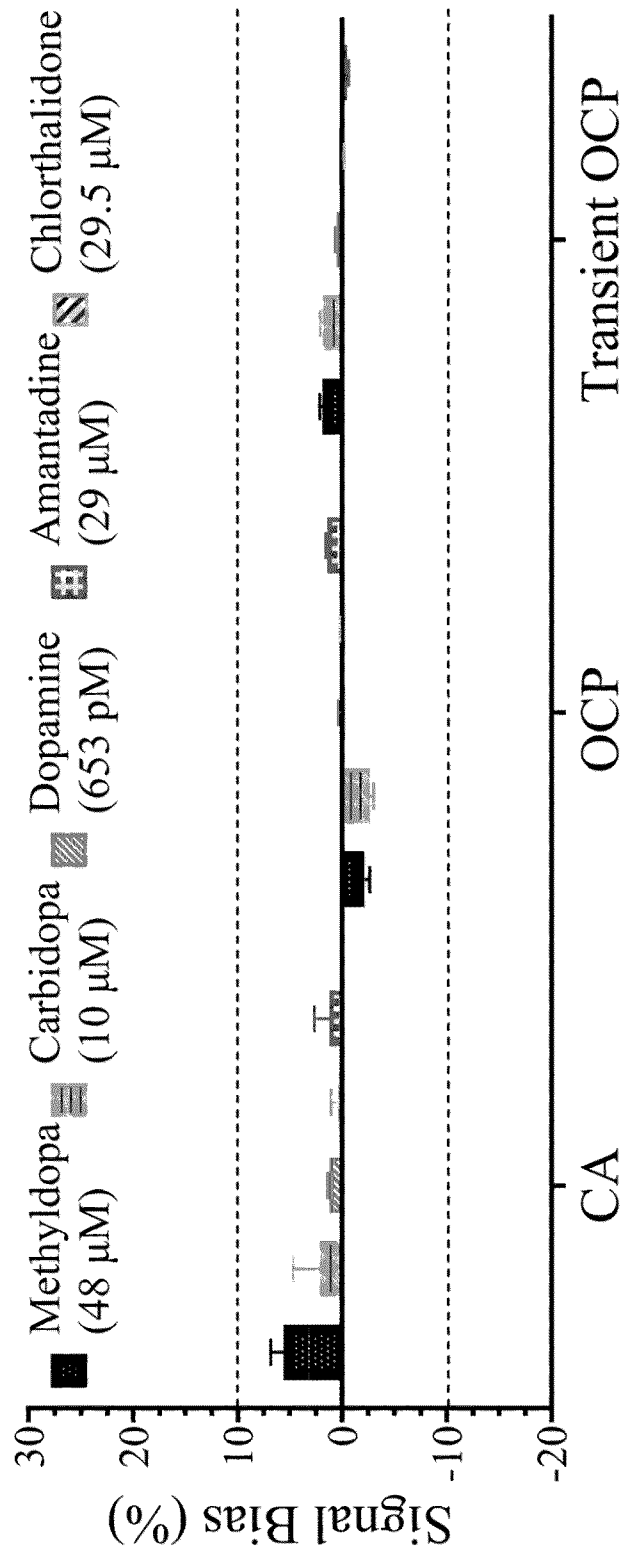


Fig. 15

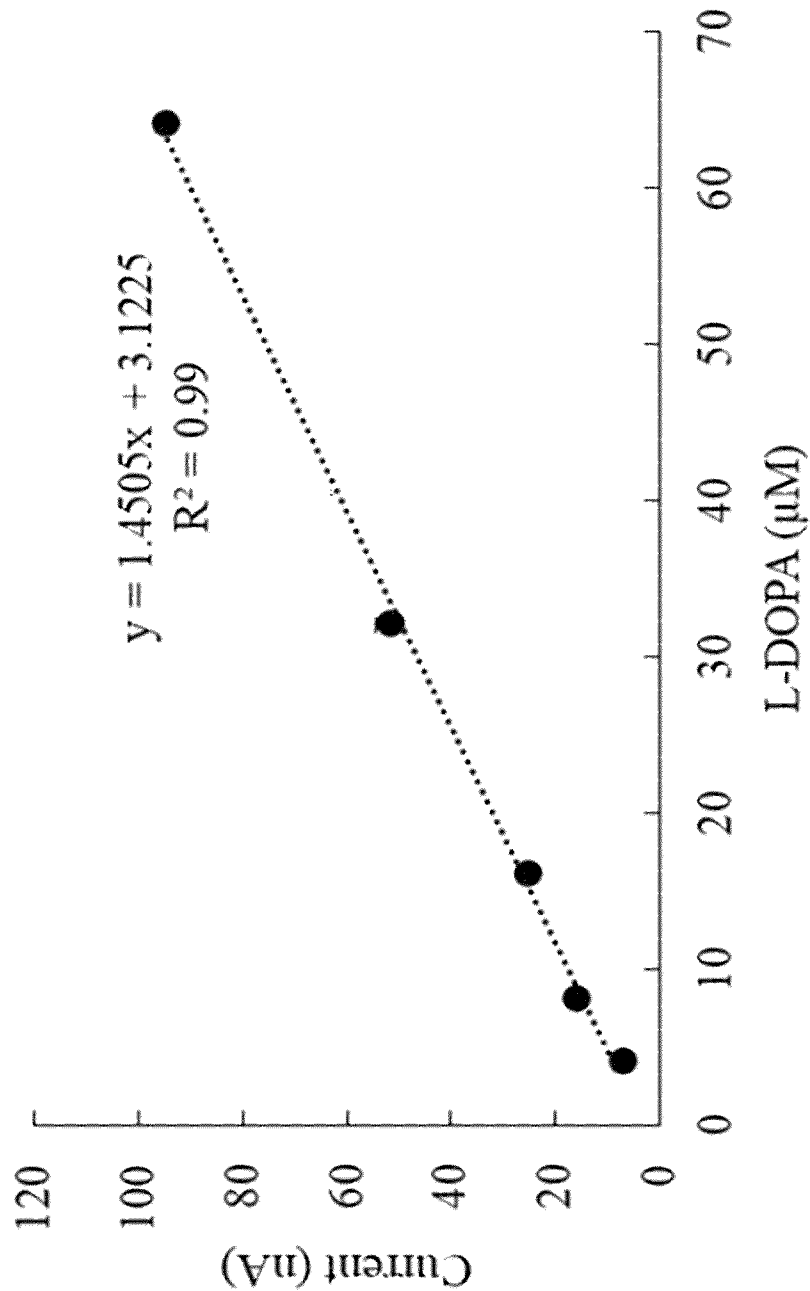


Fig. 16A

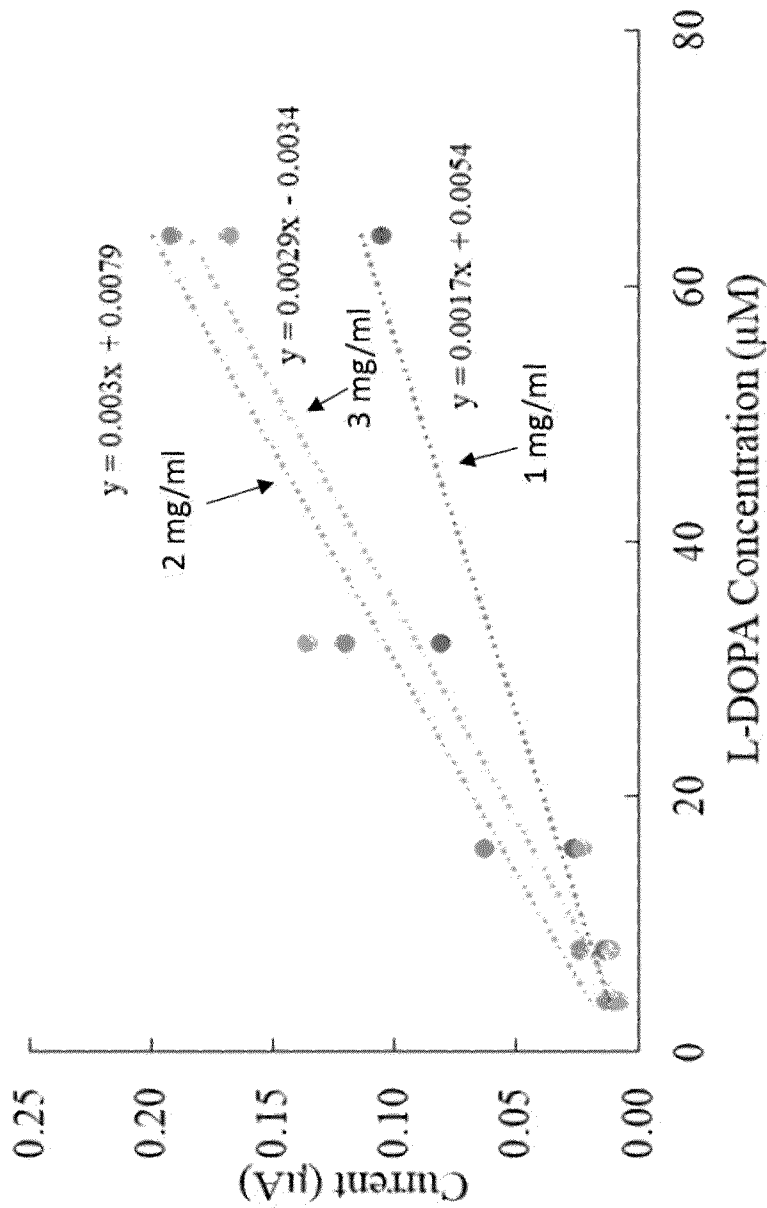


Fig. 16B

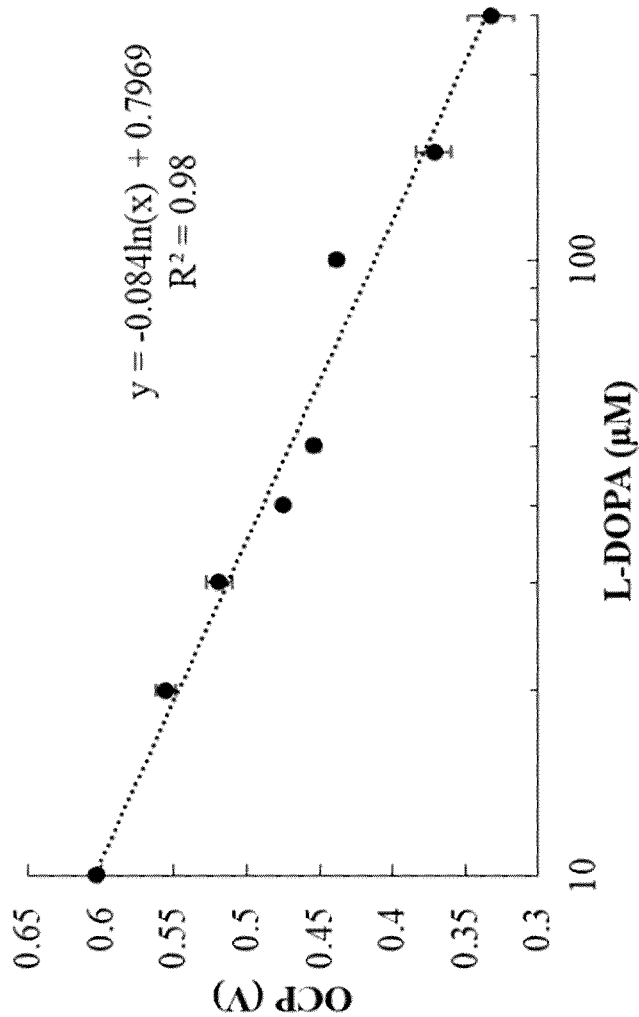


Fig. 16C

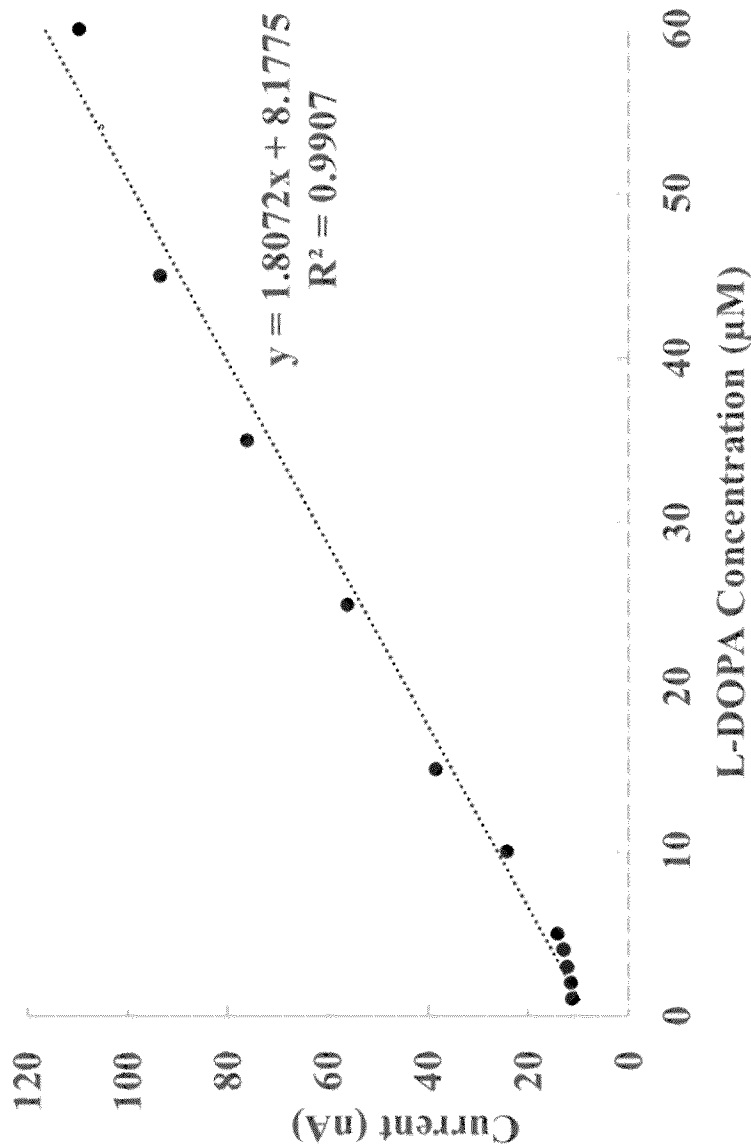


Fig. 16D

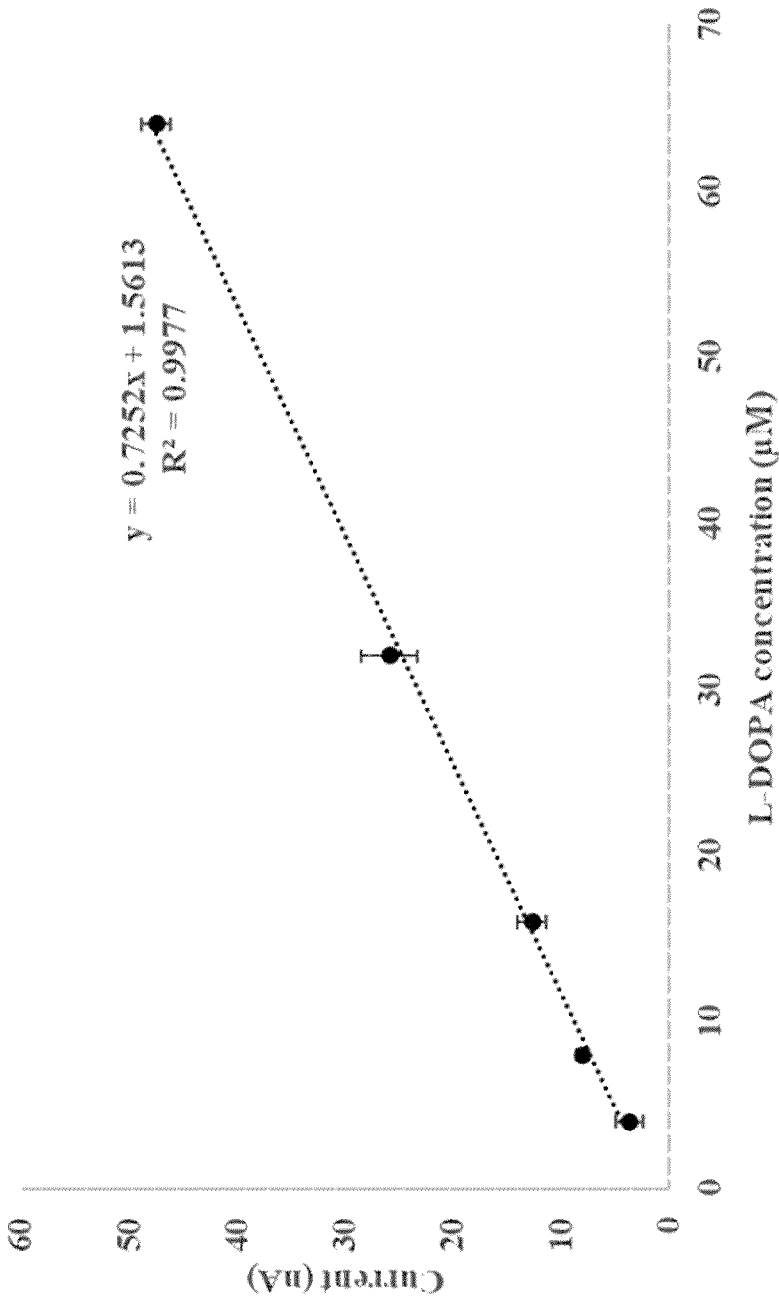


Fig. 16E

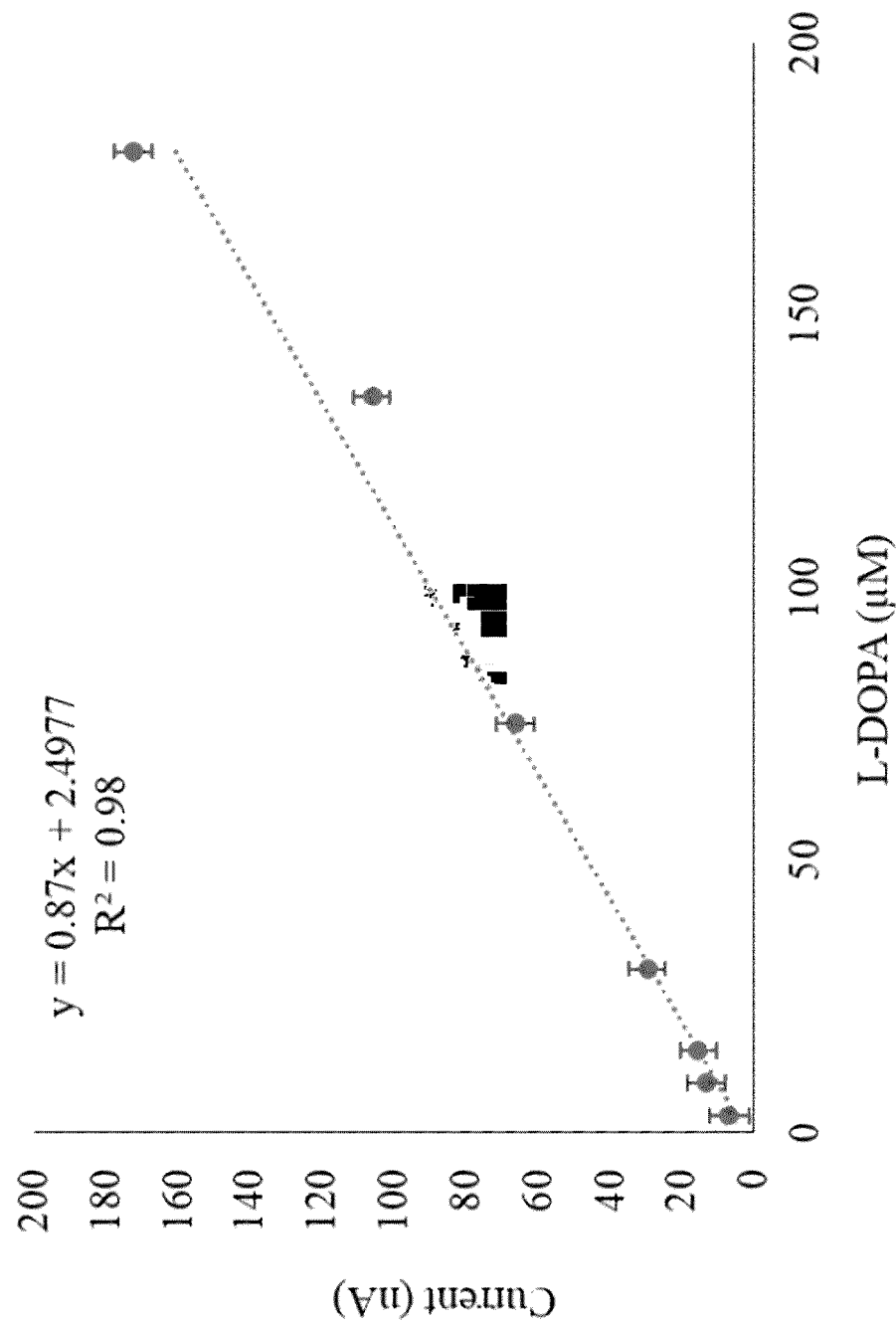


Fig. 17

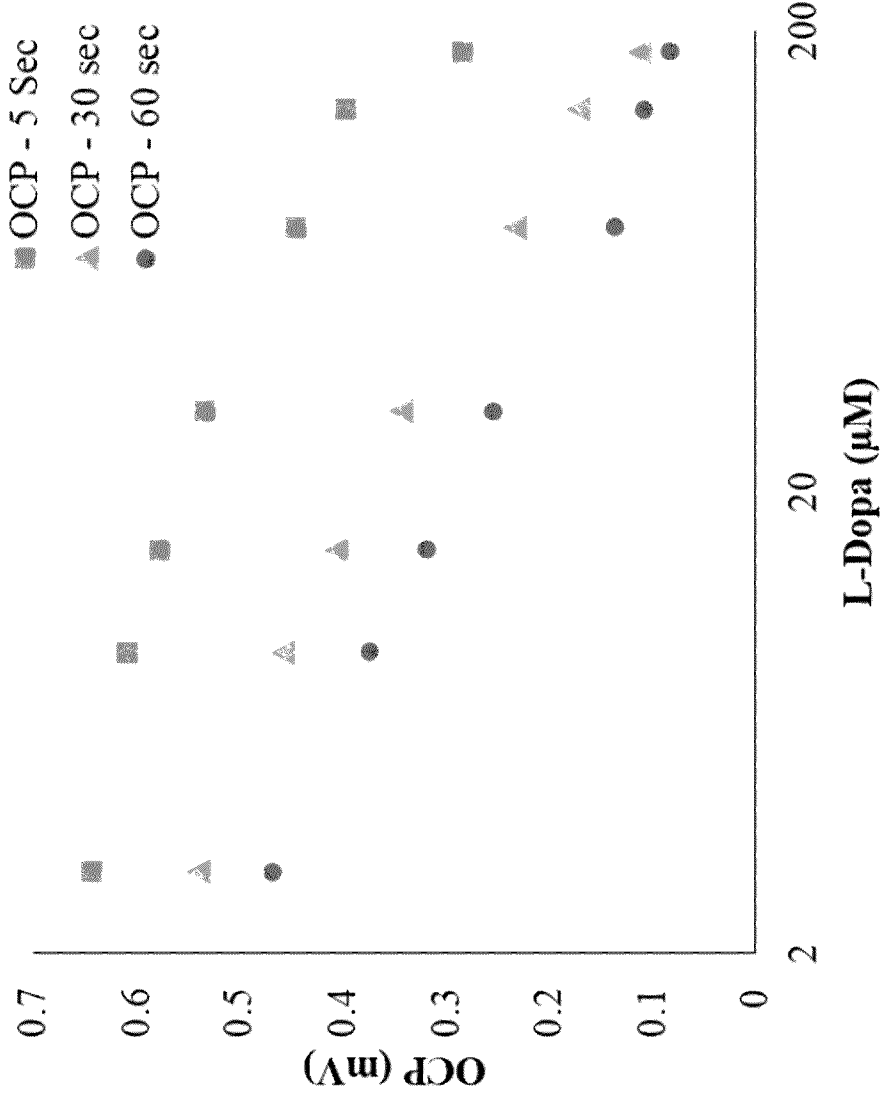


Fig. 18

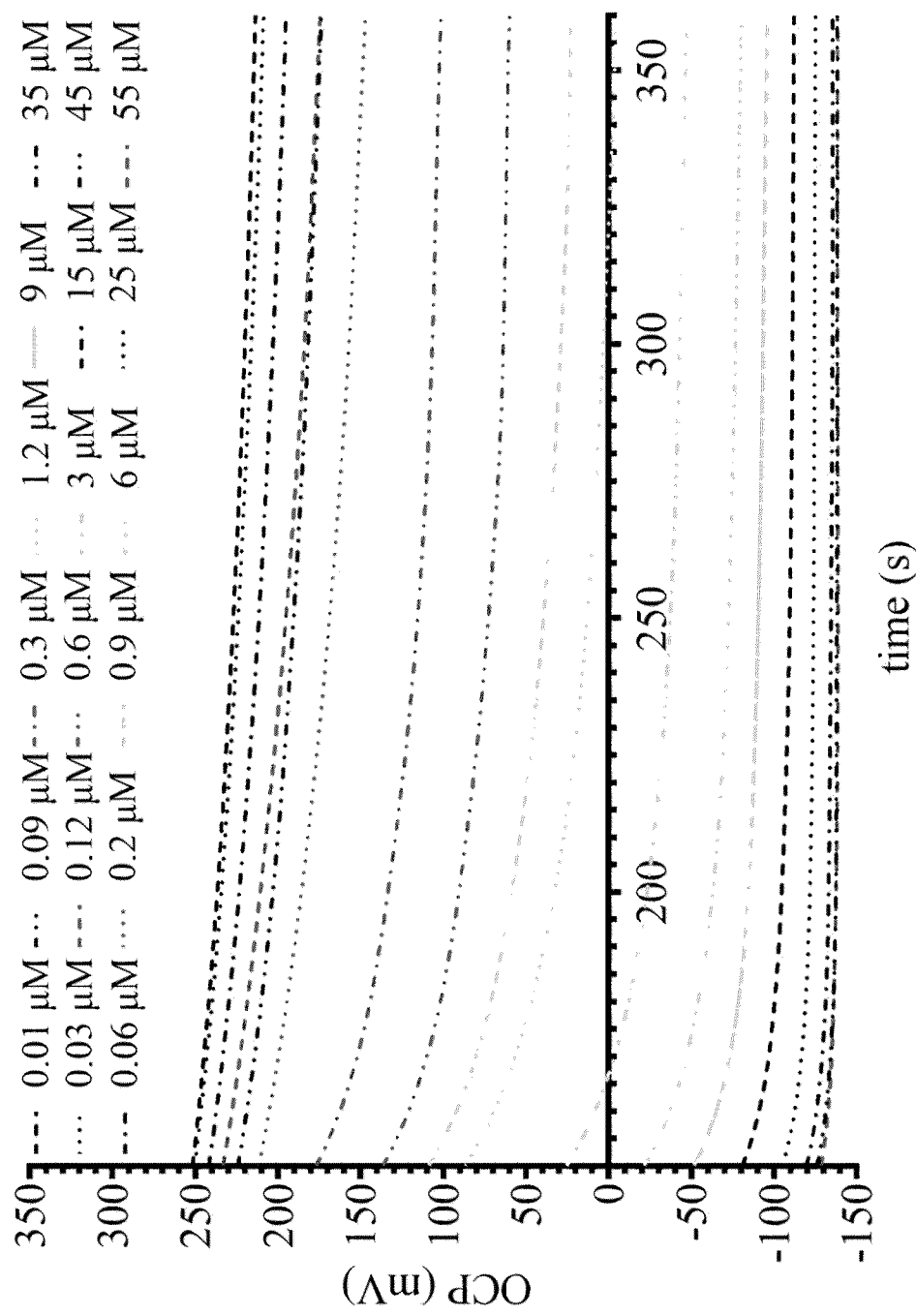


Fig. 19A

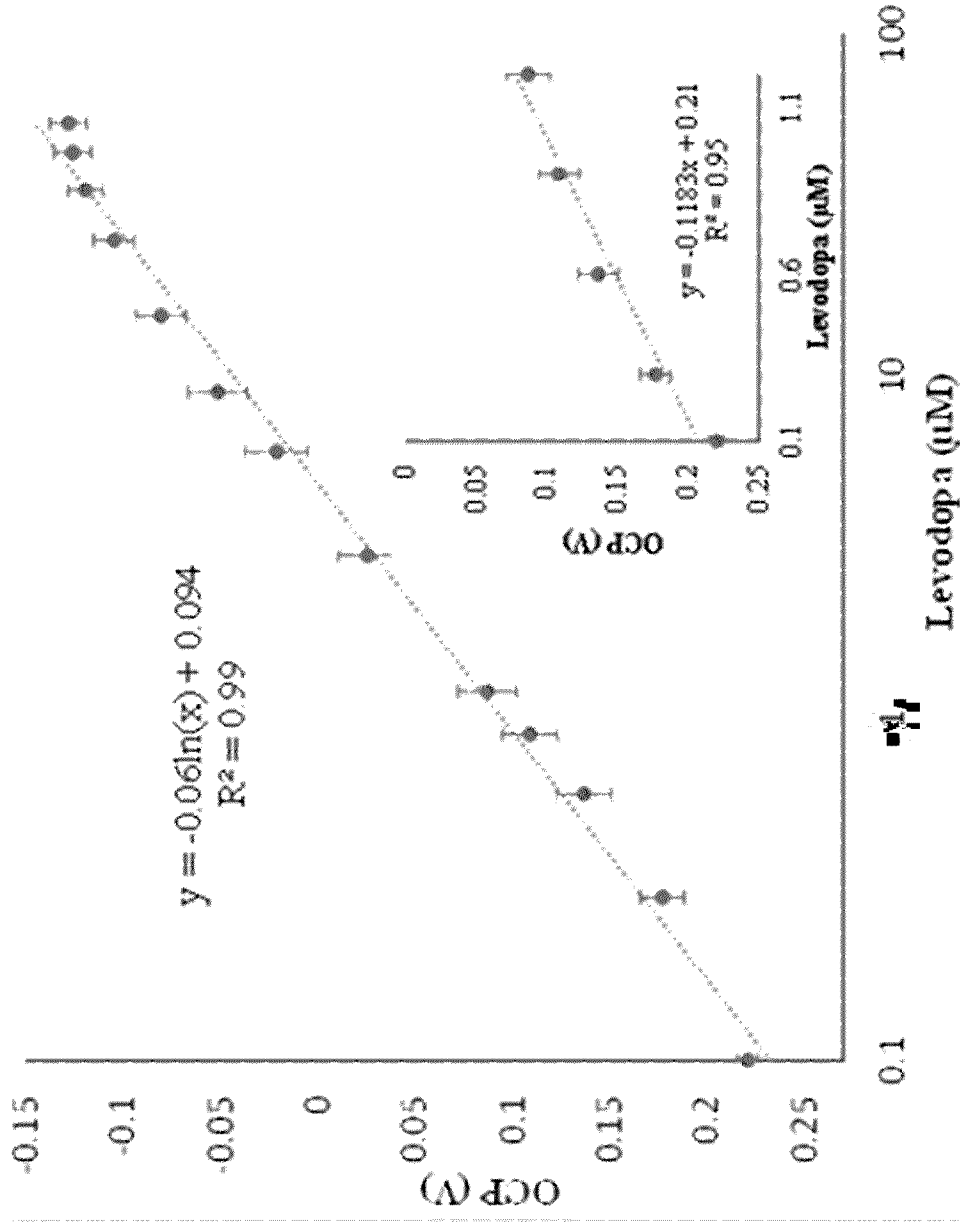


Fig. 19B

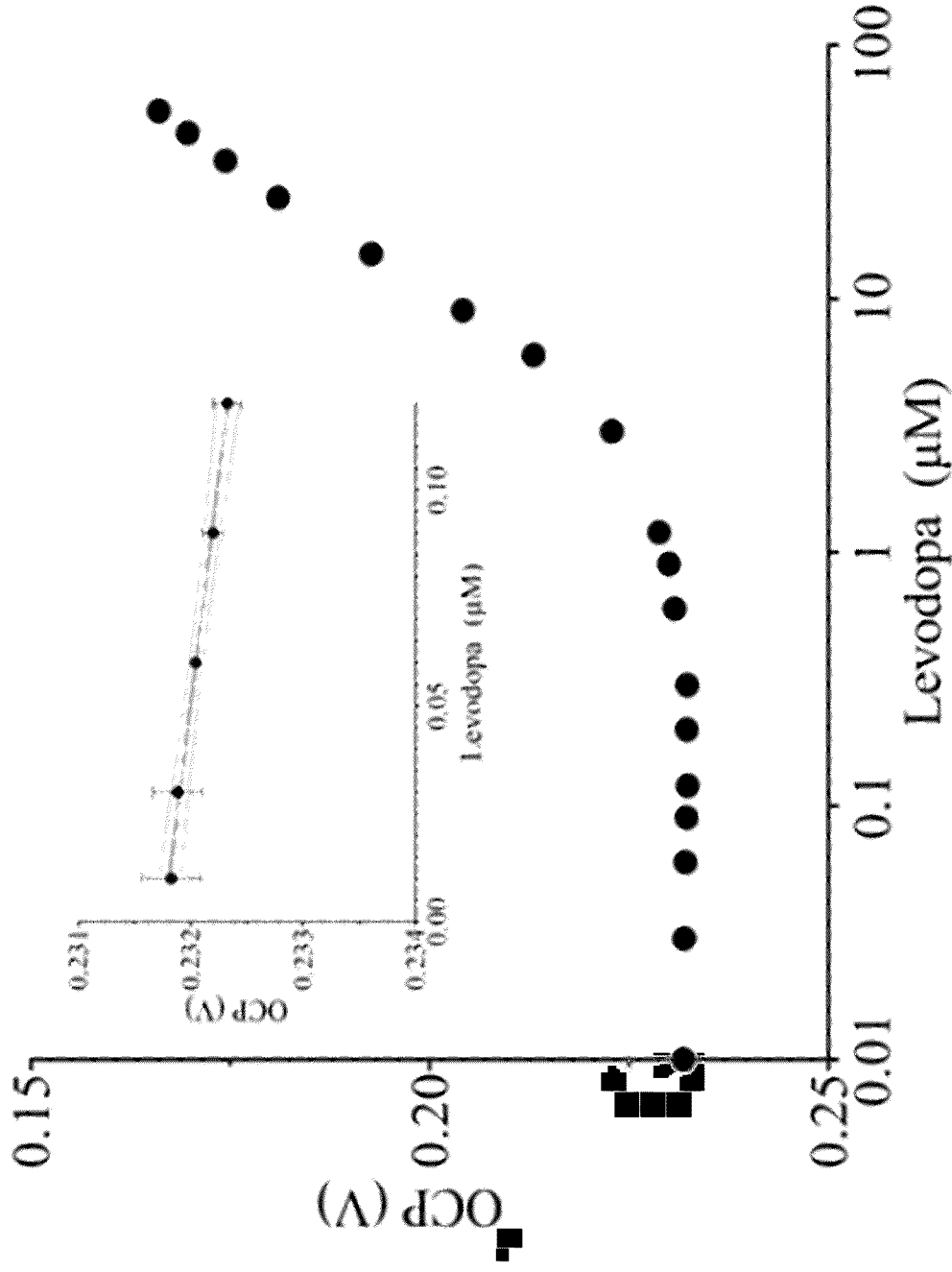


Fig. 19C

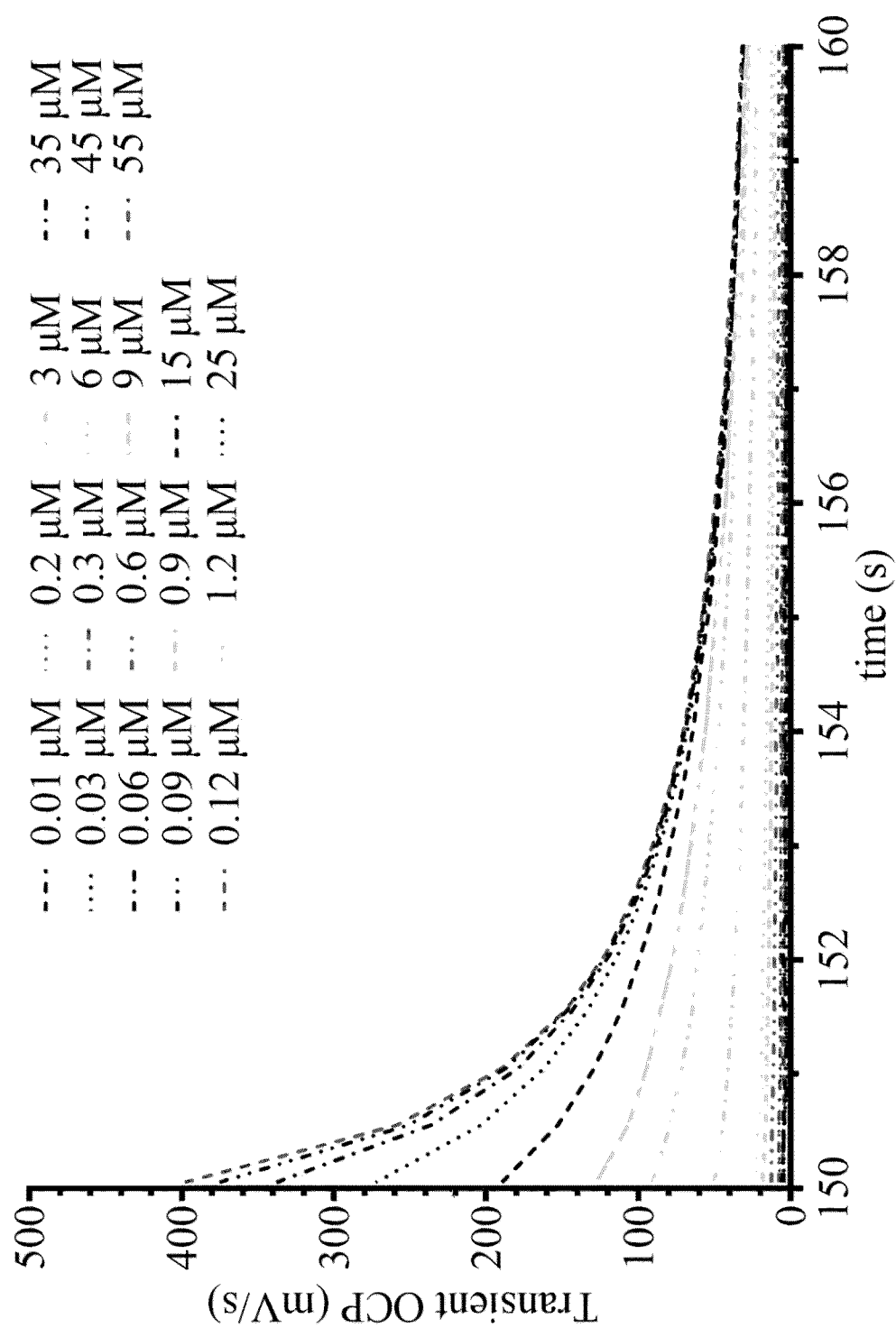


Fig. 20A

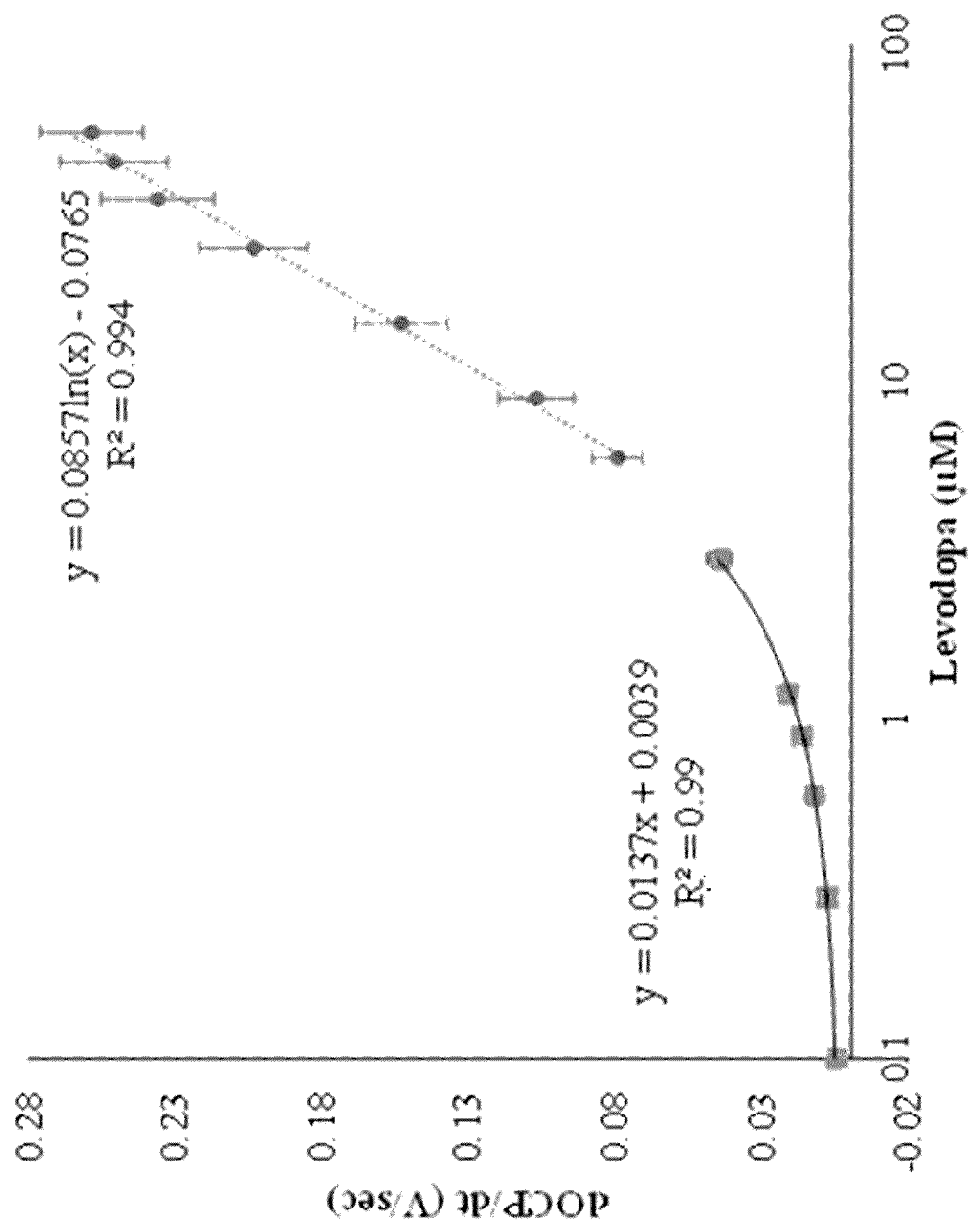


Fig. 20B

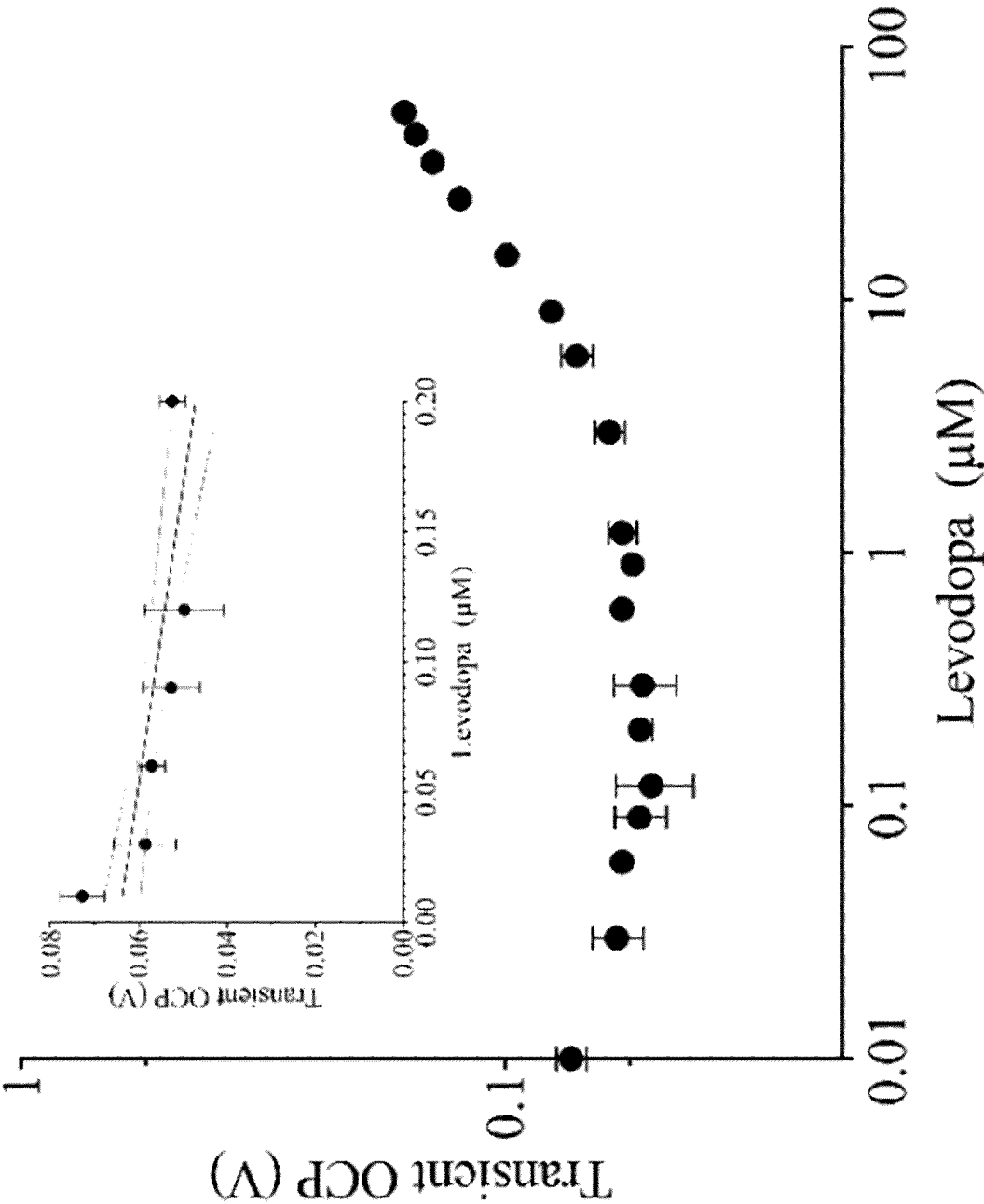


Fig. 20C

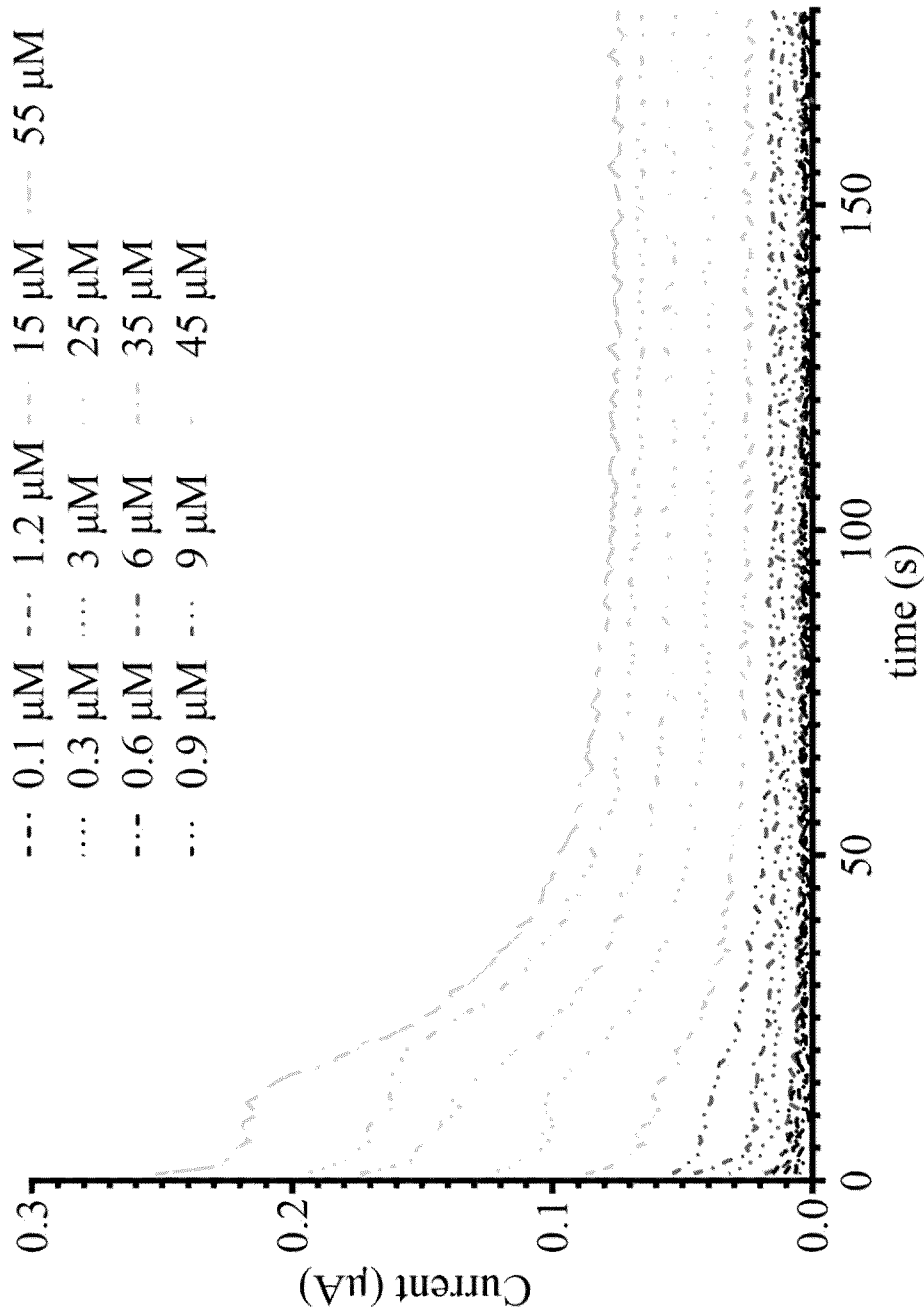


Fig. 21A

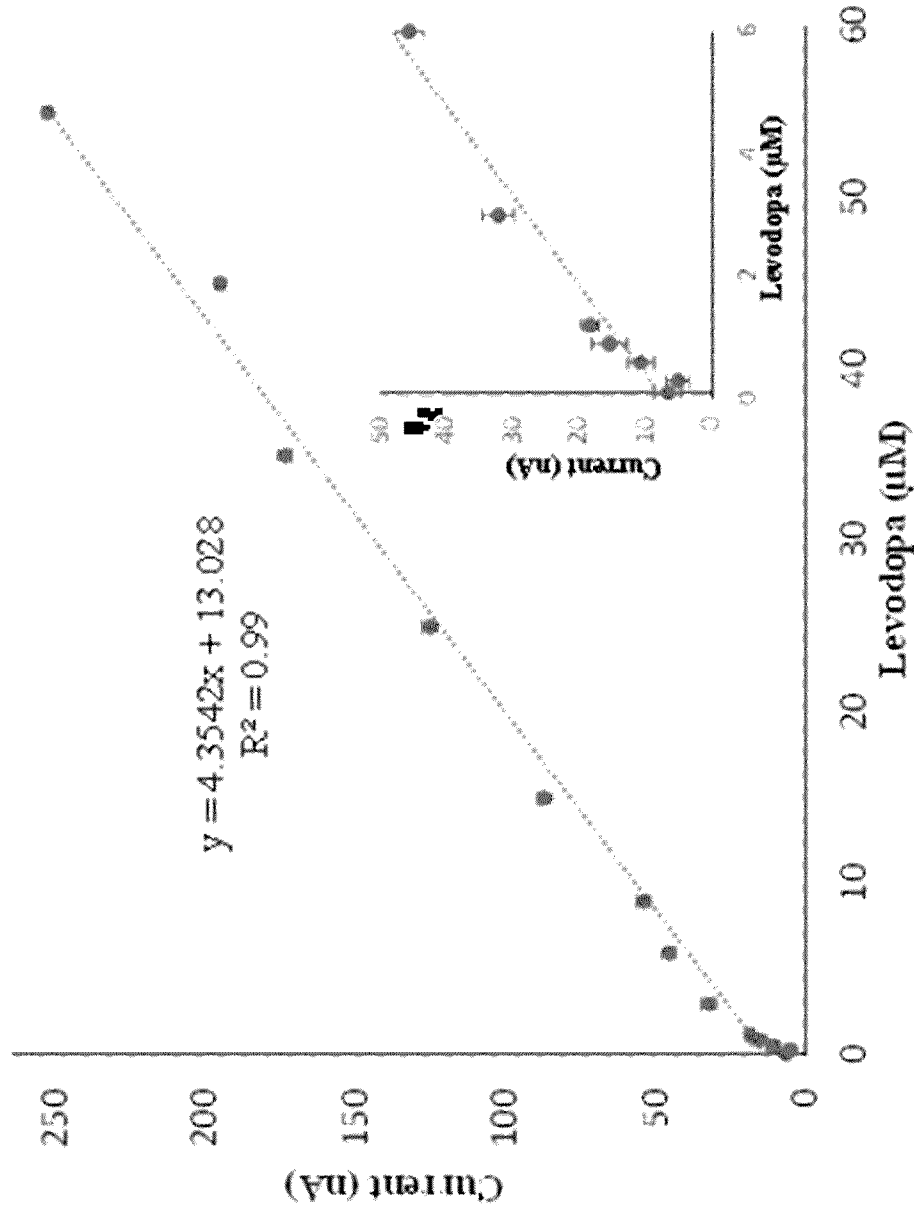


Fig. 21B

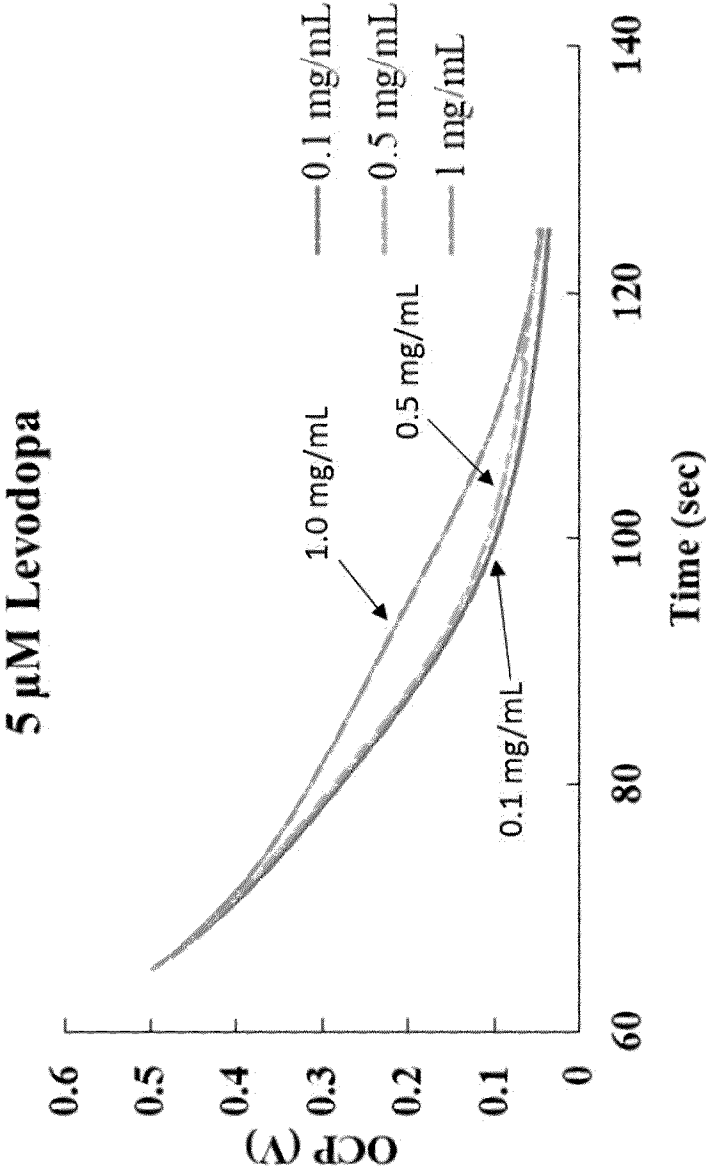


Fig. 22A

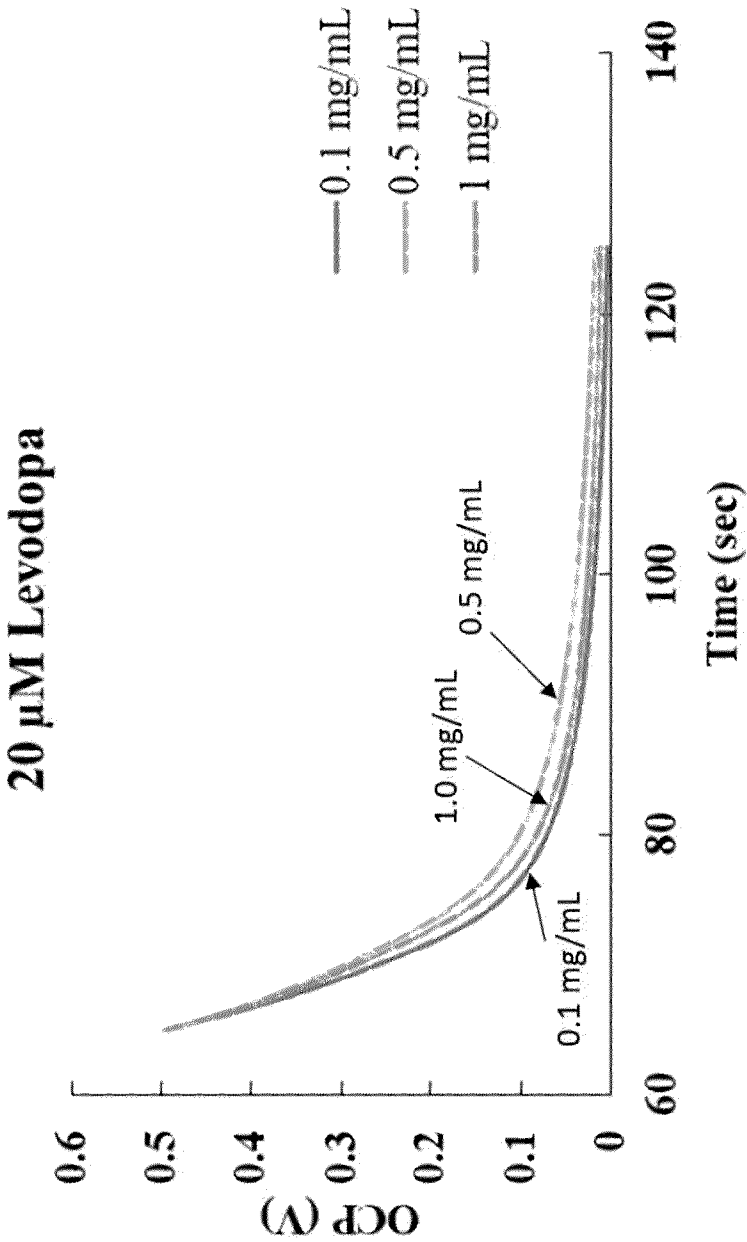


Fig. 22B

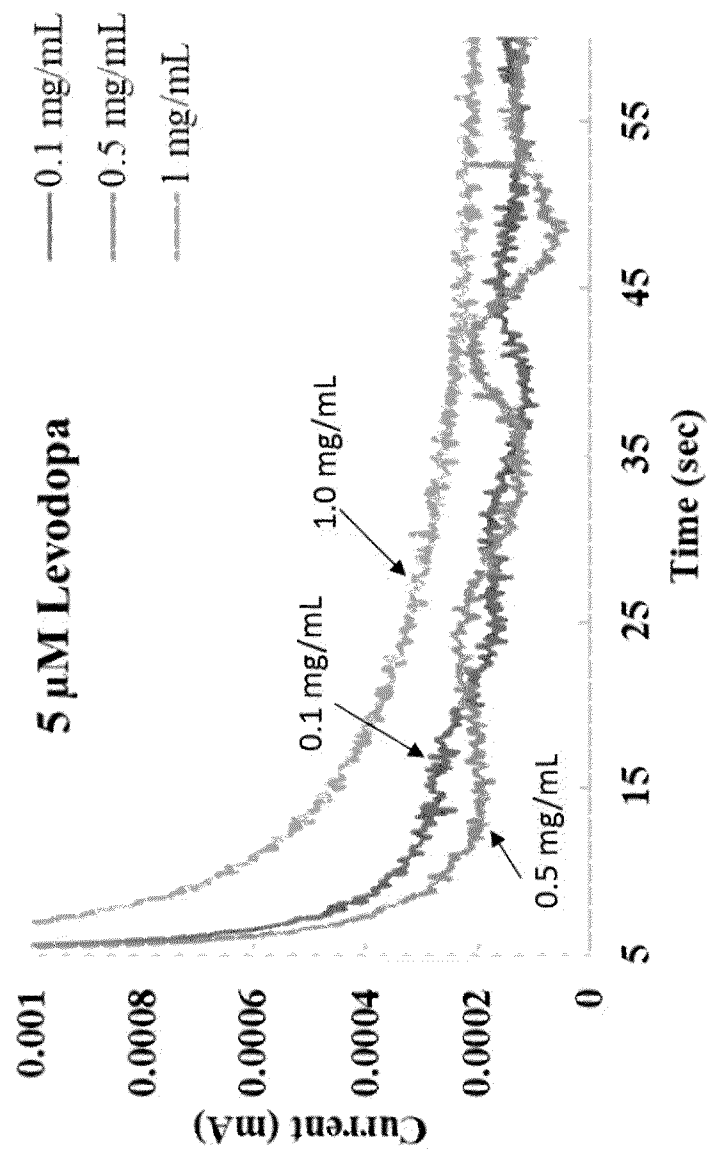


Fig. 22C

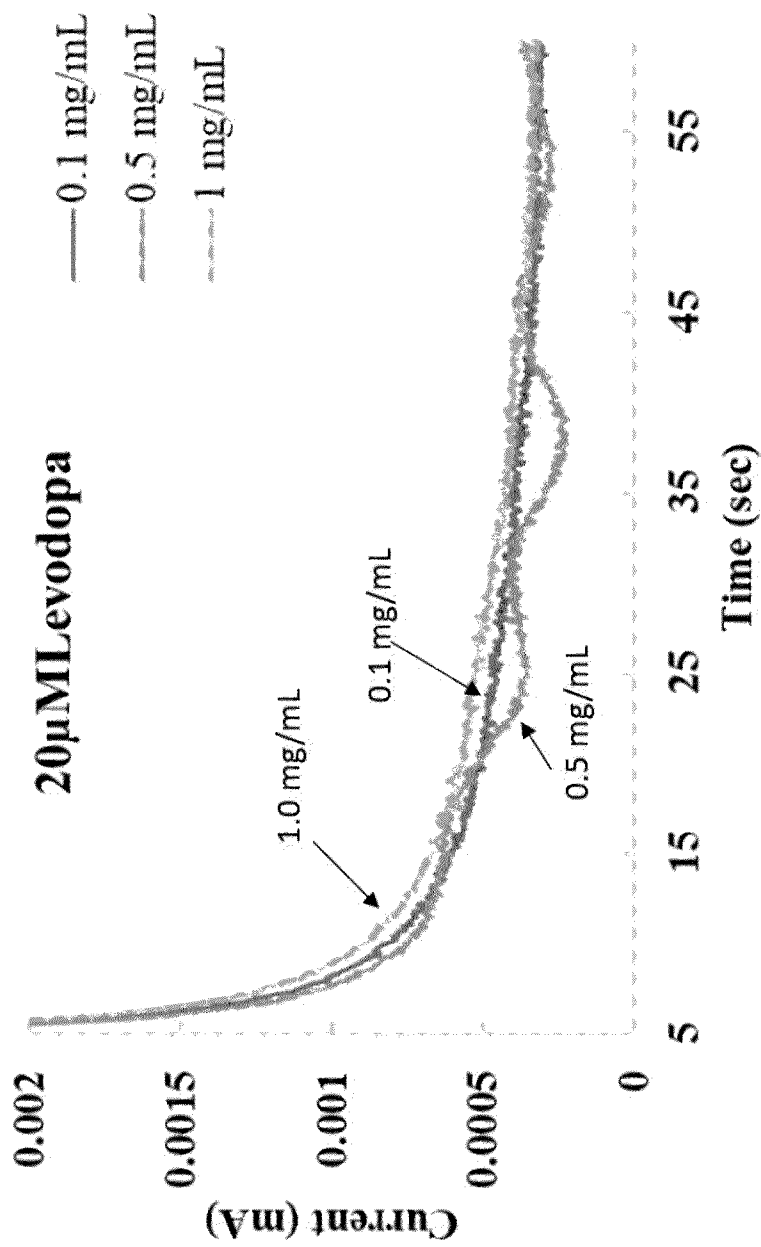


Fig. 22D

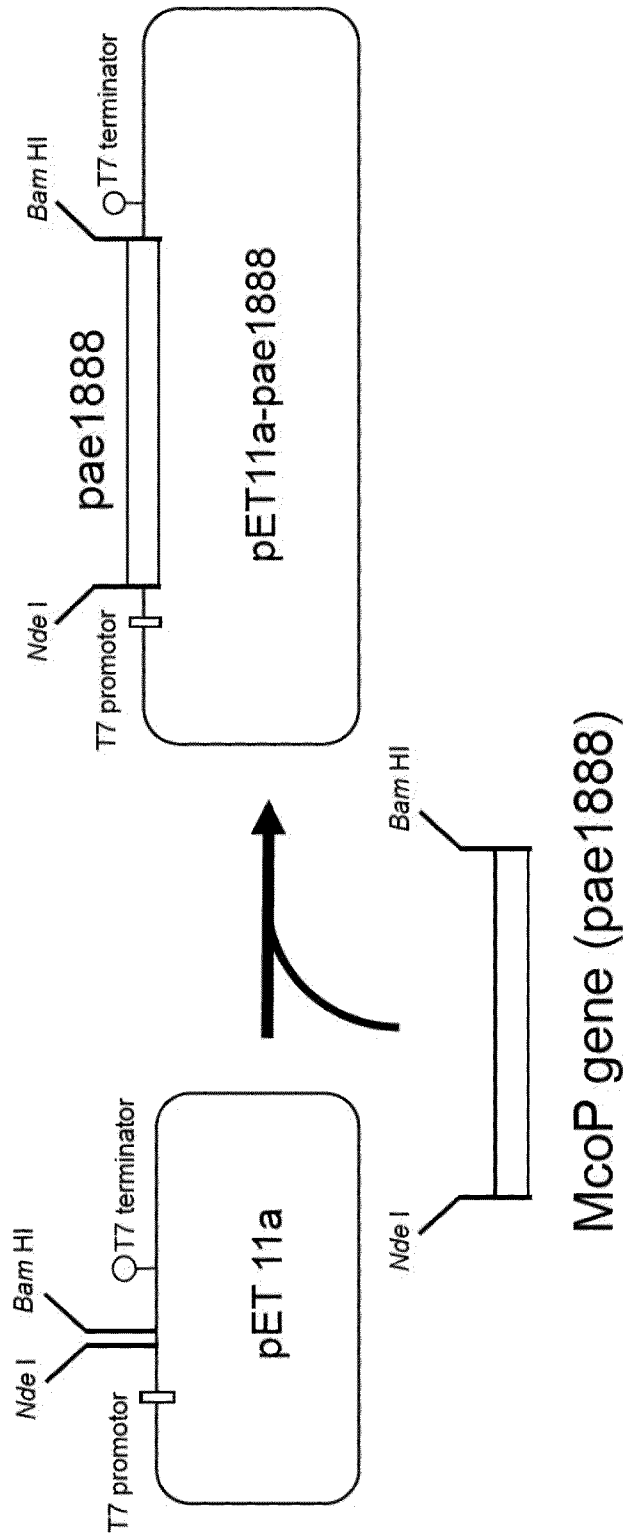


Fig. 23

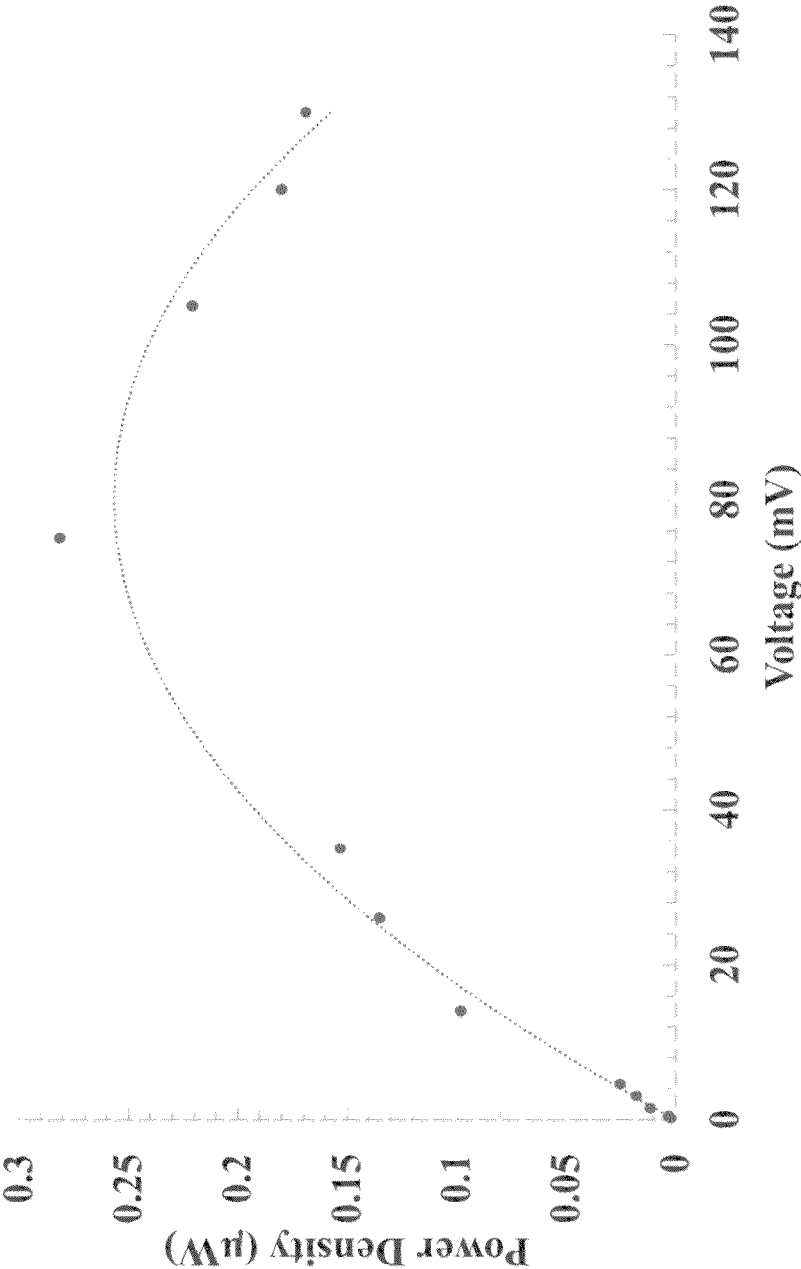


Fig. 24A

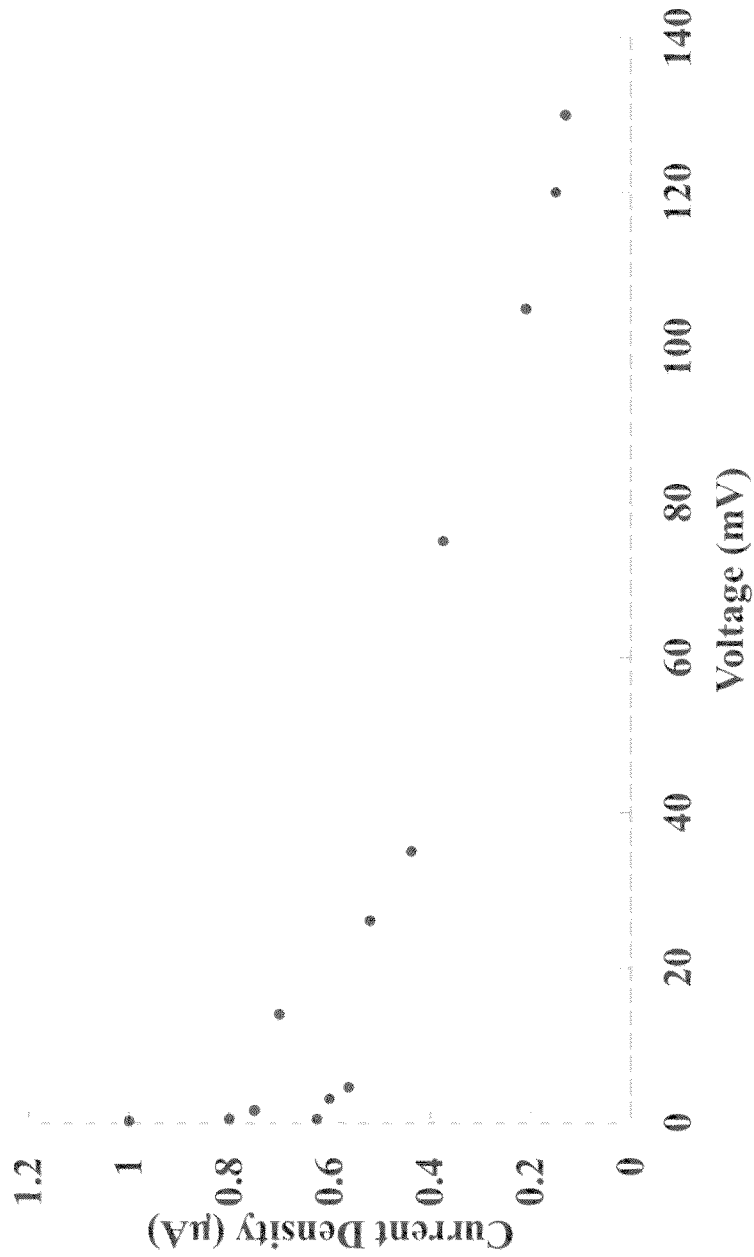


Fig. 24B

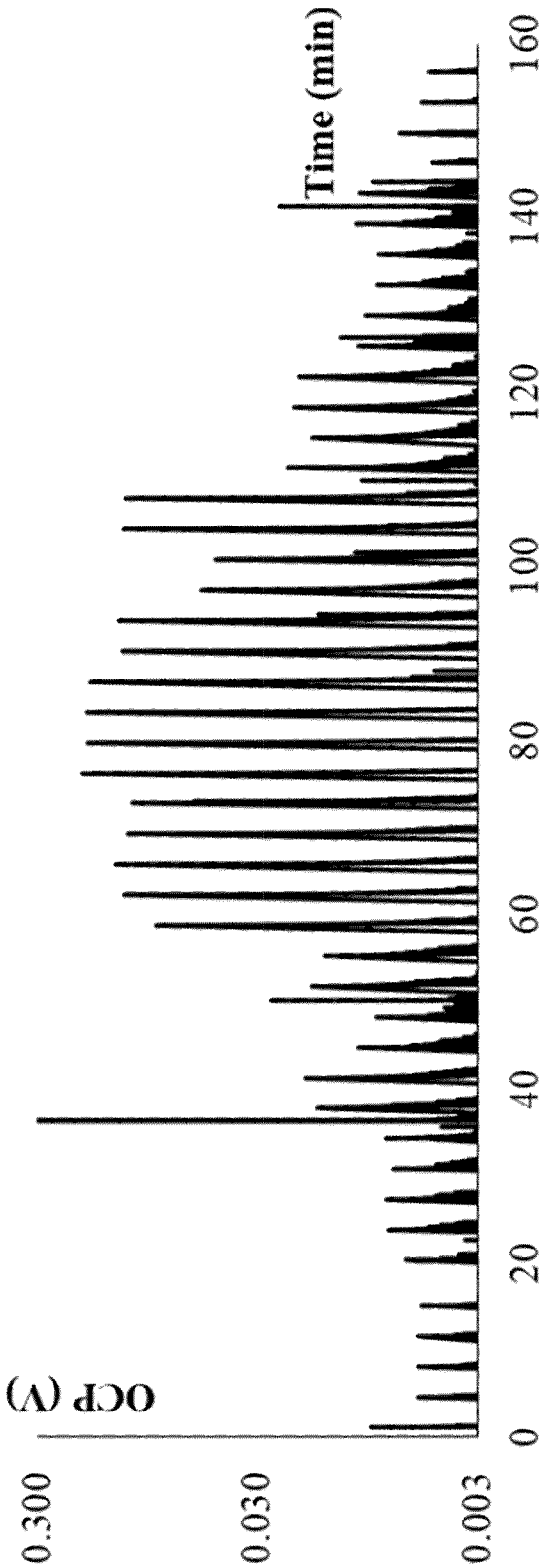


Fig. 25A

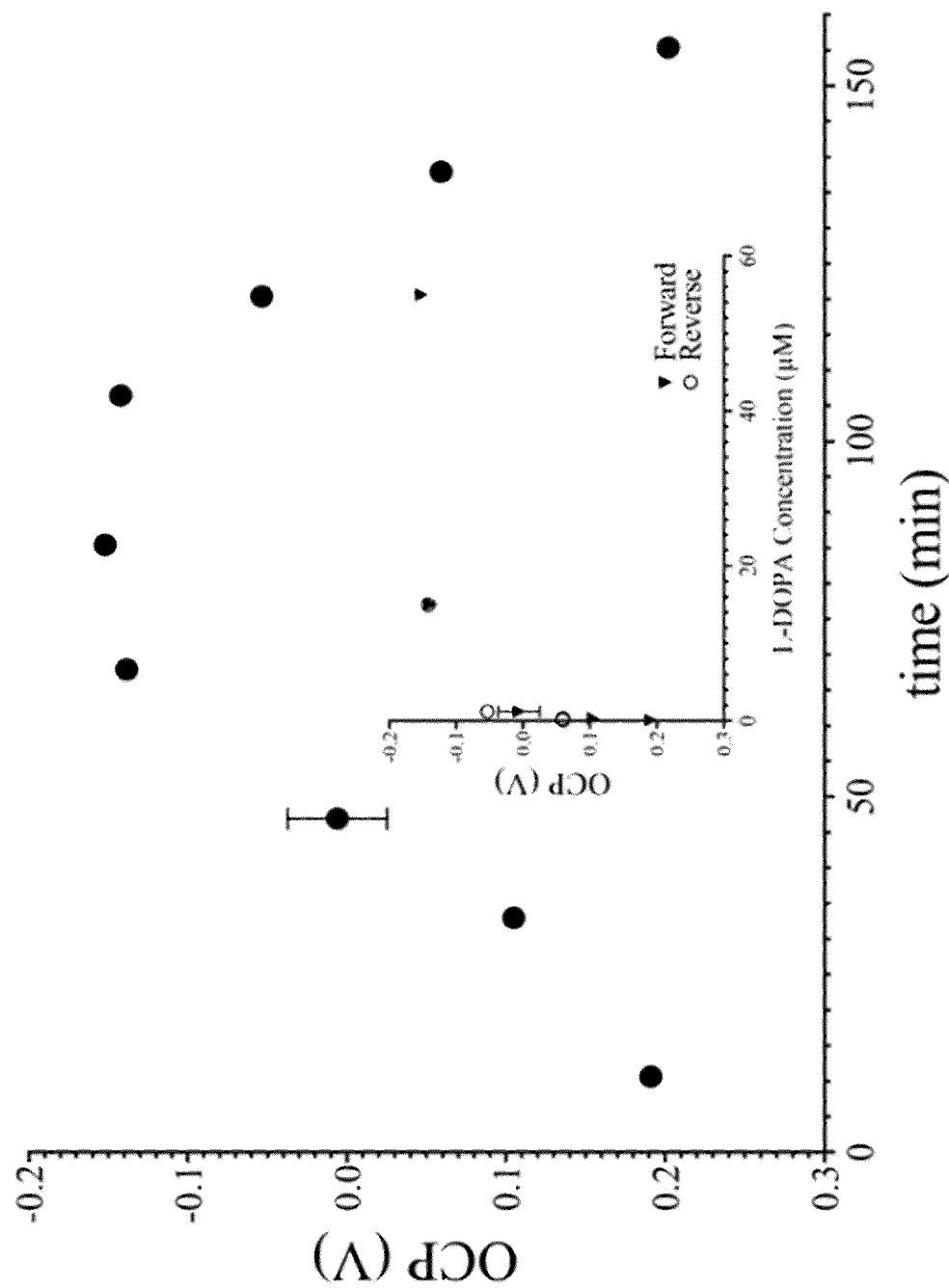


Fig. 25B

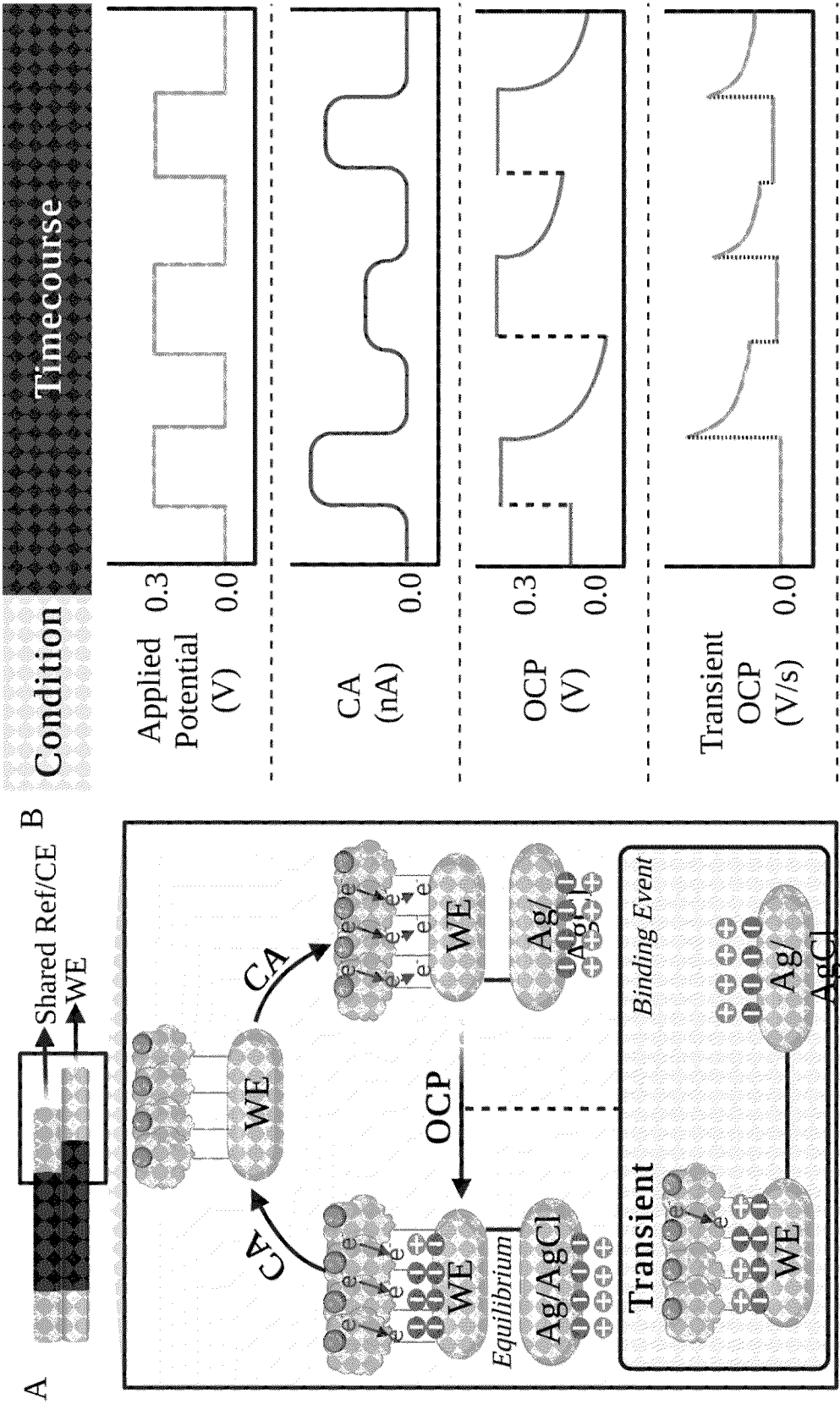


Fig. 26B

Fig. 26A

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Fig. 27A

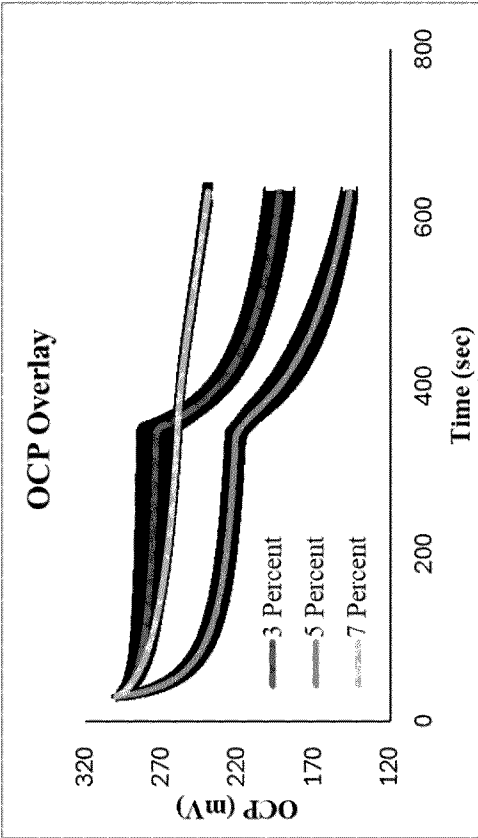


Fig. 27B

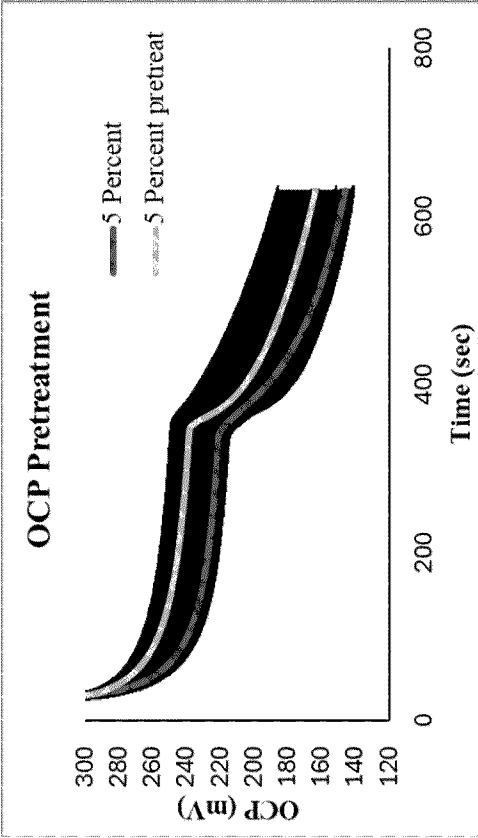


Fig. 27C

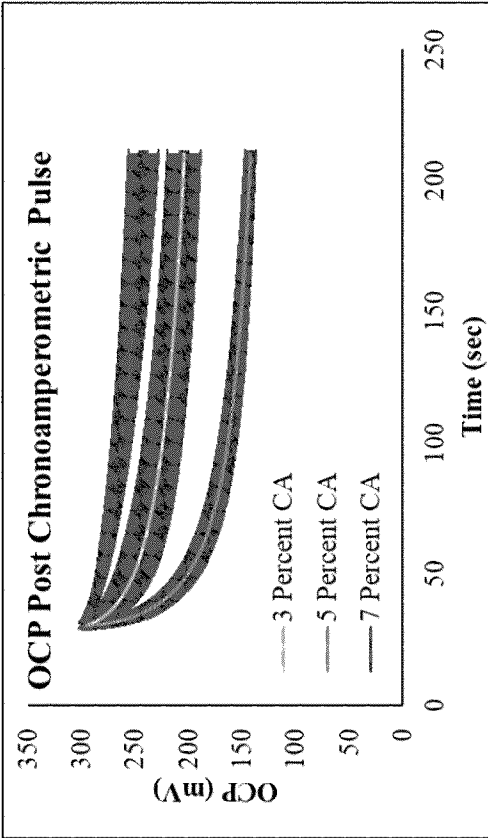


Fig. 27D

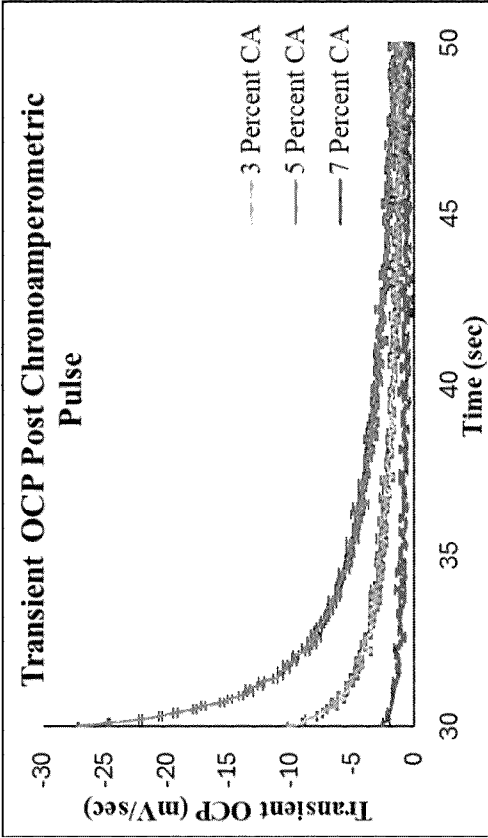


Fig. 28A

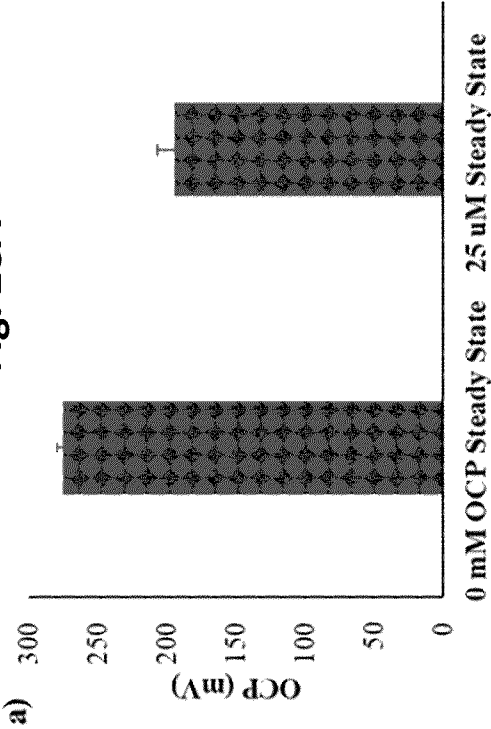


Fig. 28B

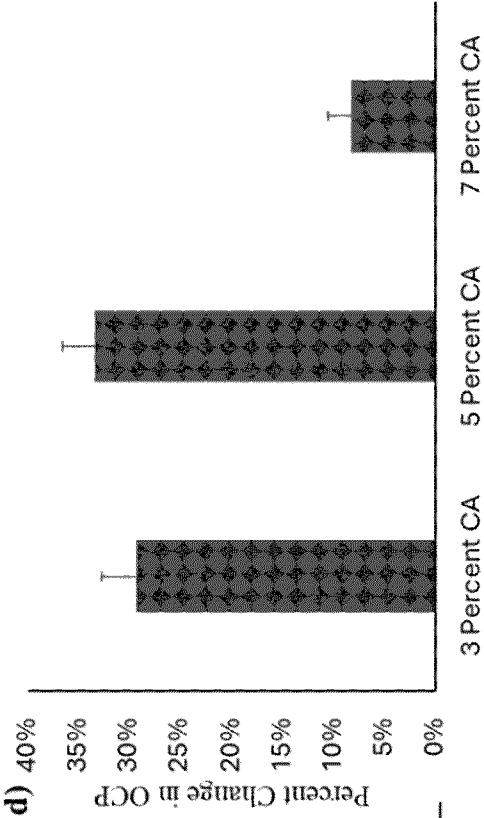
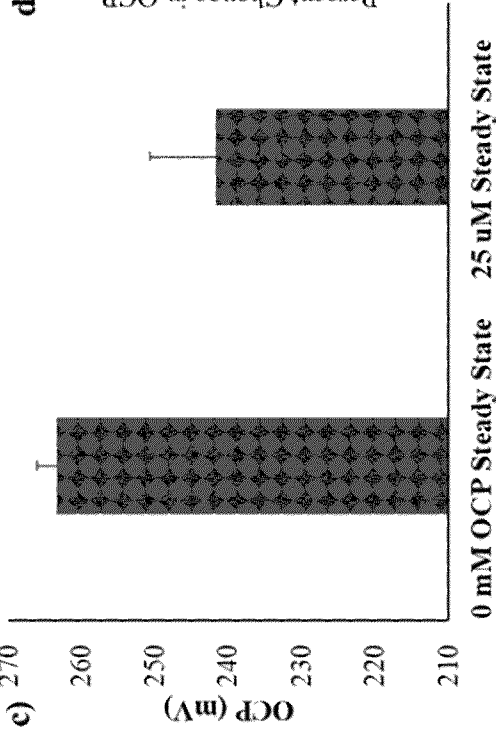
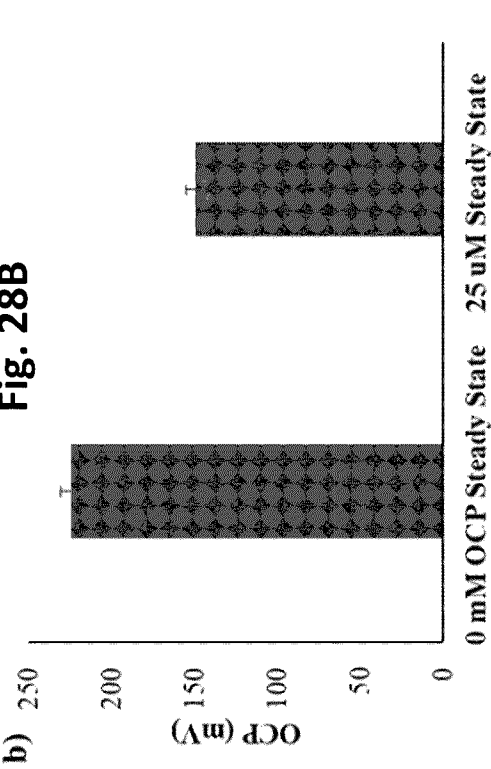


Fig. 28C

Fig. 28D

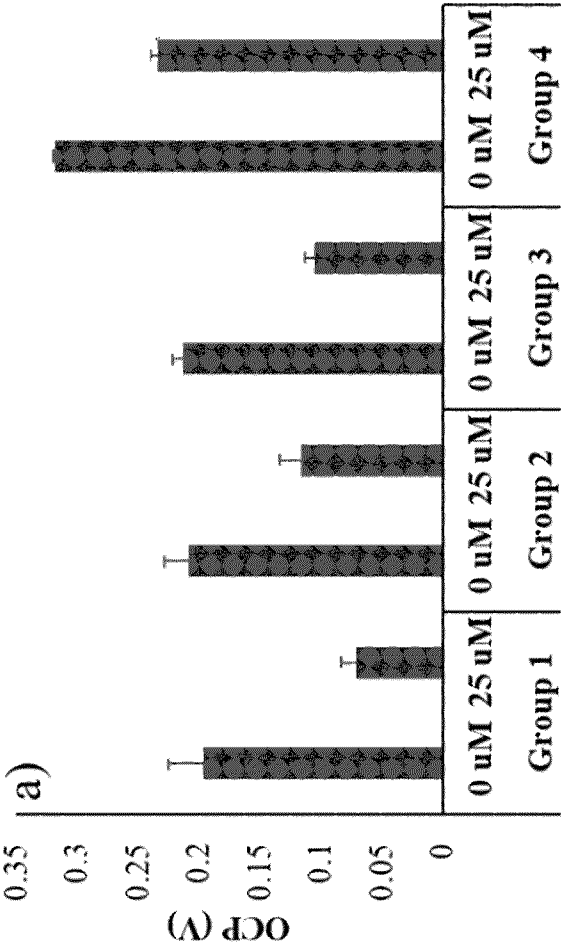


Fig. 29A

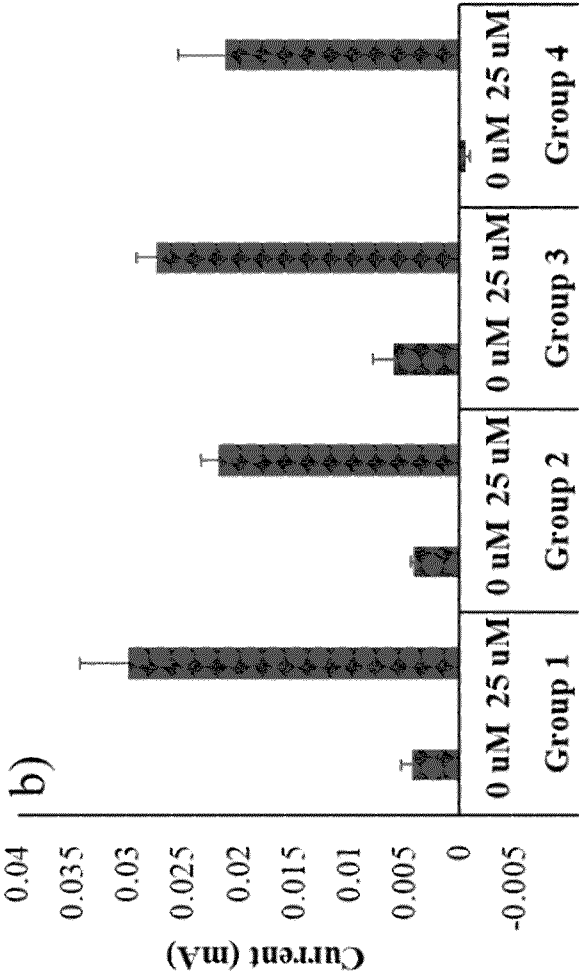


Fig. 29B

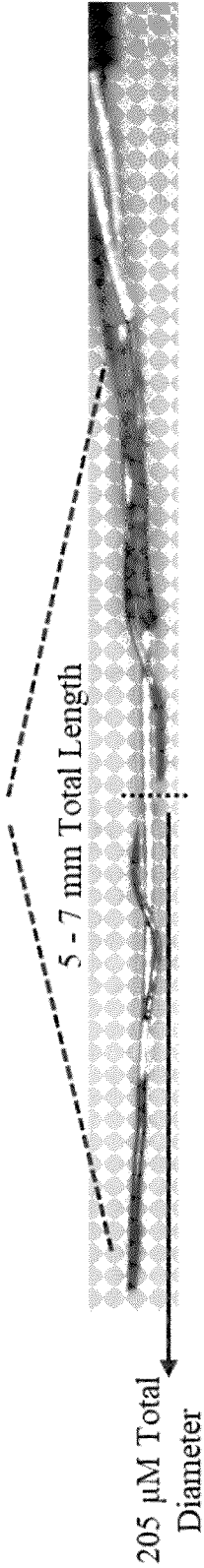


Fig. 30

Fig. 31A

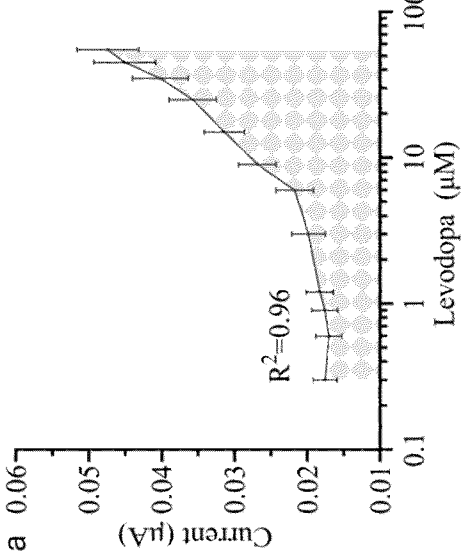


Fig. 31B

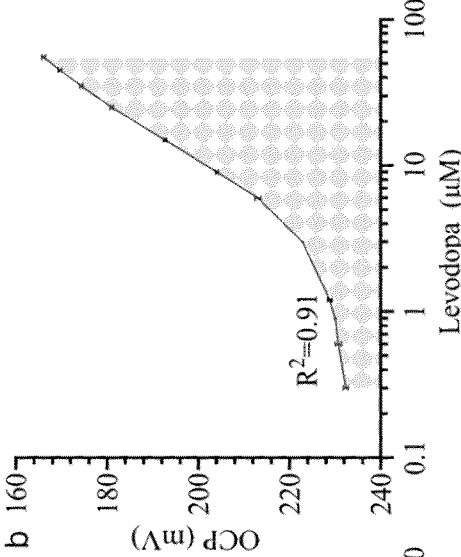
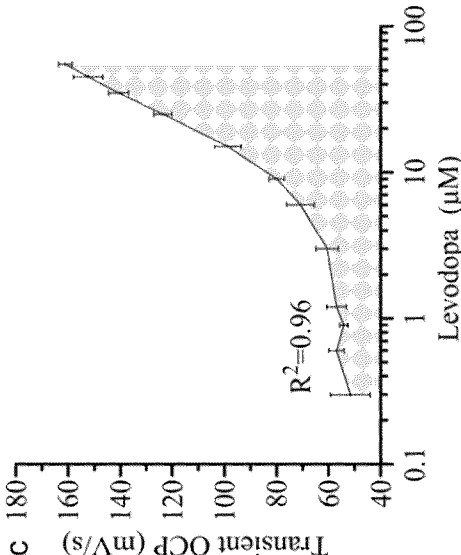


Fig. 31C



d Chronoamperometry

	Buffer	Plasma
LOD	138 nM	8.51 µM
Sensitivity	$4.28 \frac{nA}{\mu M}$	$0.58 \frac{nA}{\mu M}$

e Open Circuit Potential

	Buffer	Plasma
LOD	72.8 nM	337 nM
Sensitivity	$56.6 \frac{mV}{\mu M}$	$13.8 \frac{mV}{\mu M}$

f Transient Open Circuit Potential

	Buffer	Plasma
LOD	616 nM	4.99 µM
Sensitivity	$70.7 \frac{mV}{s \cdot \mu M}$	$20.1 \frac{mV}{s \cdot \mu M}$

Fig. 31D

Fig. 31E

Fig. 31F

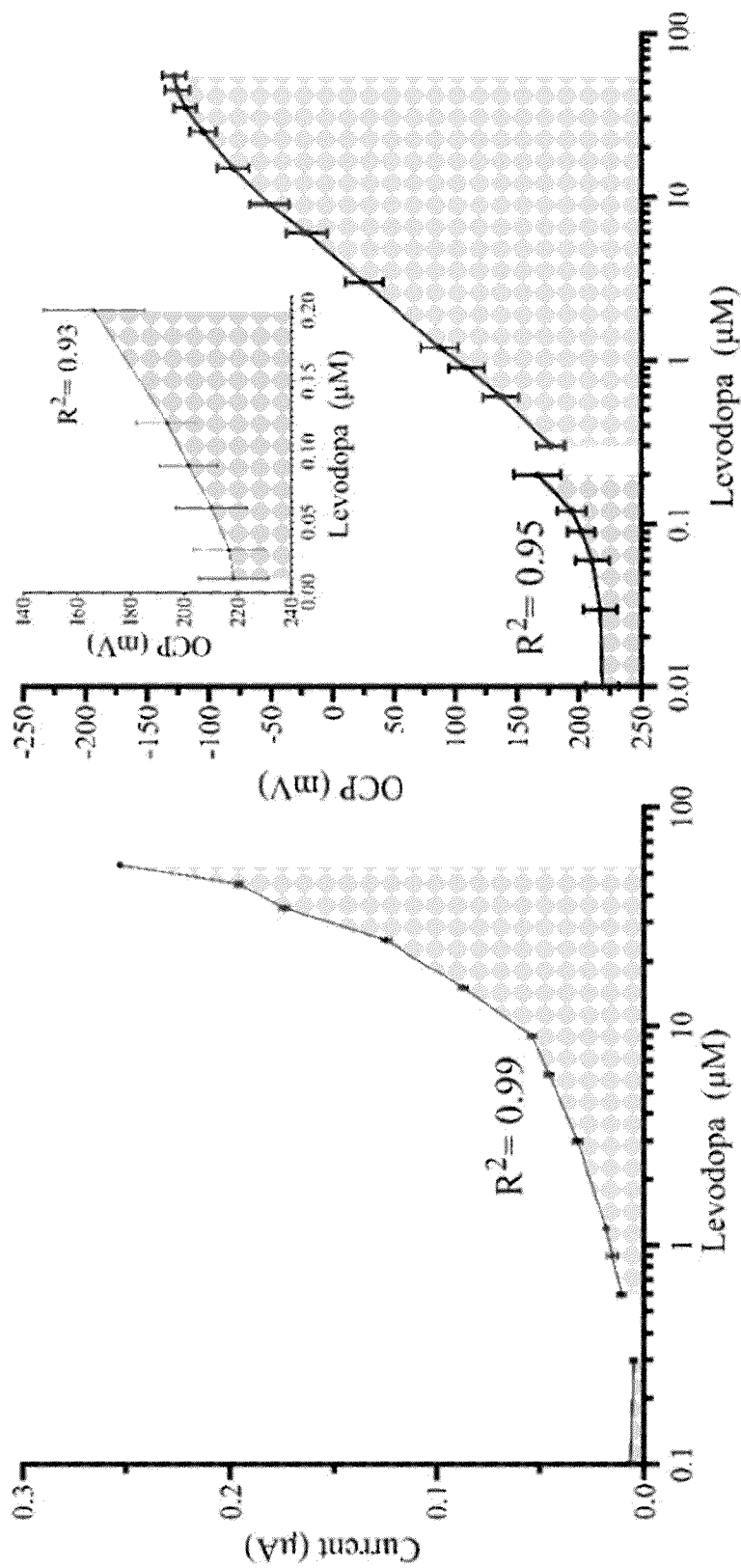


Fig. 32A

Fig. 32B

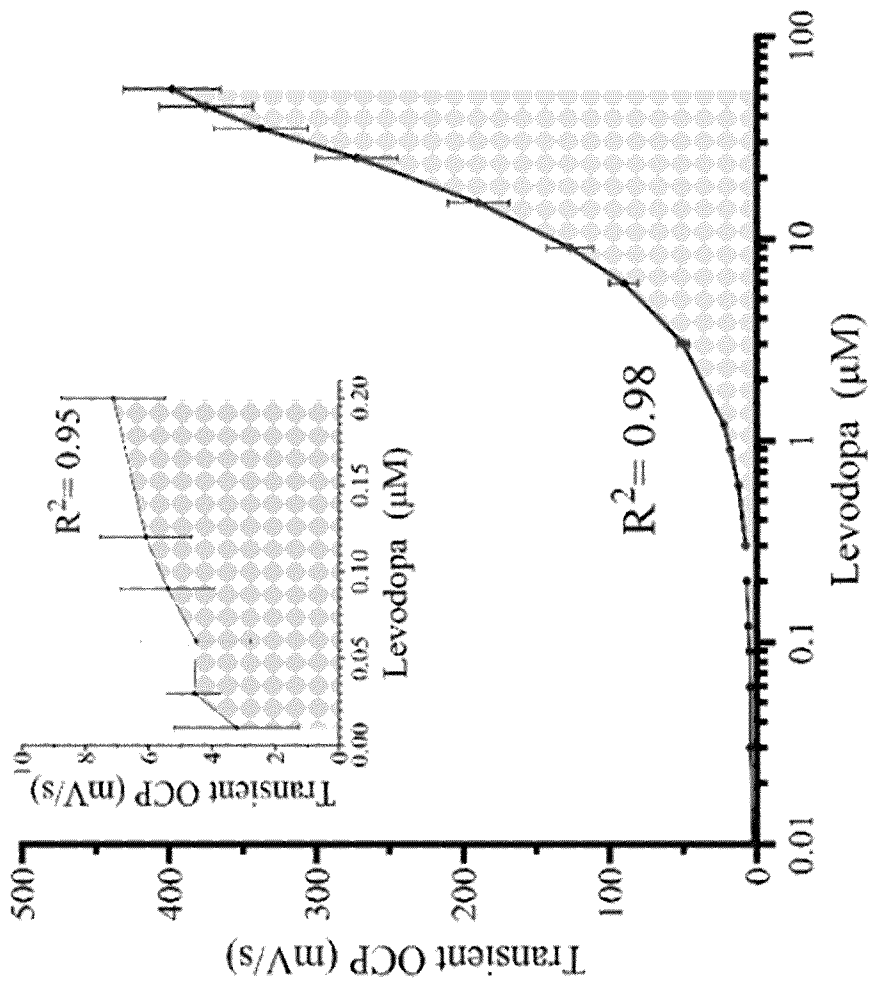


Fig. 32C

Fig. 33A

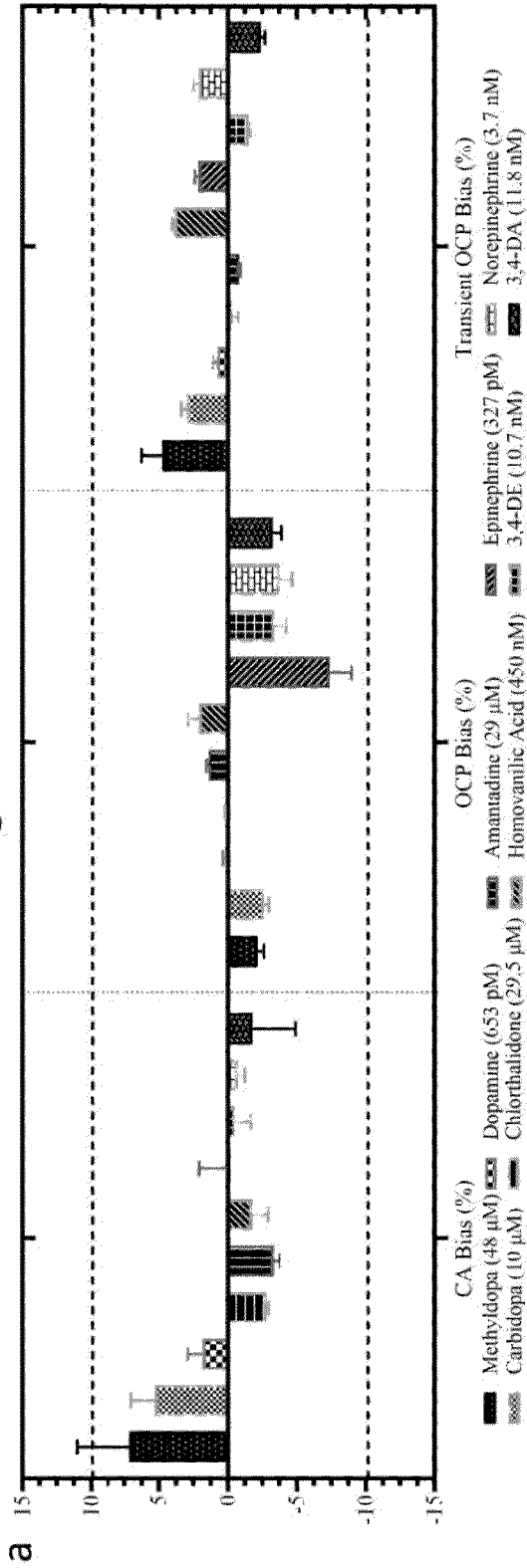
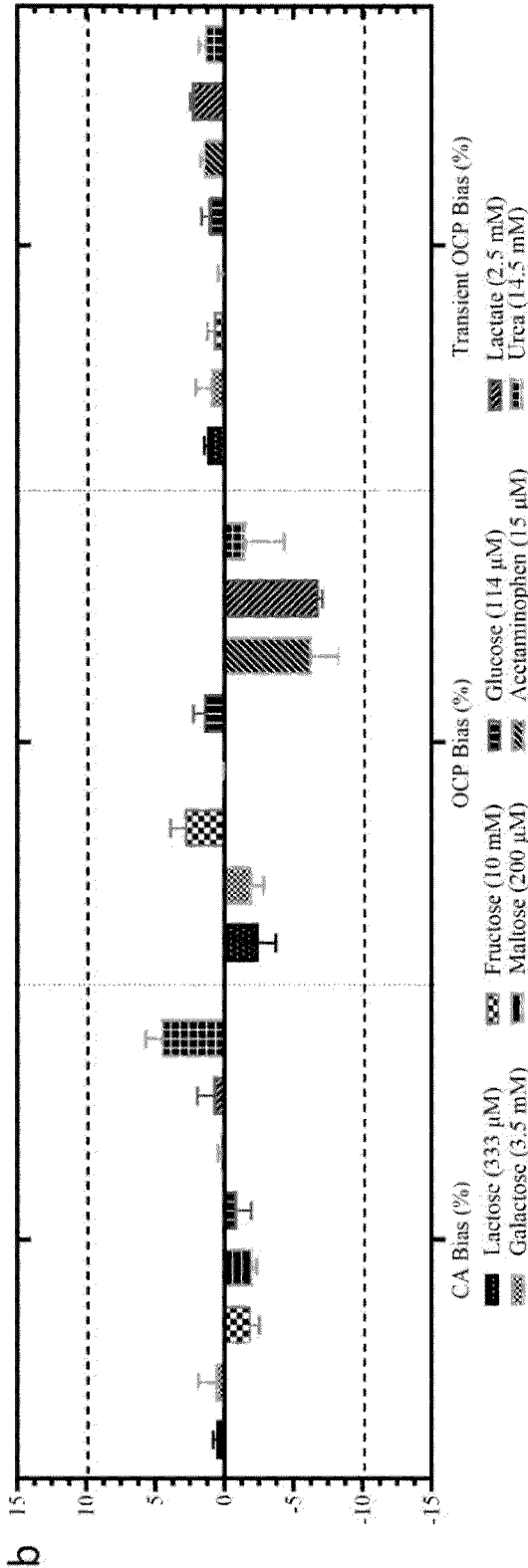


Fig. 33B



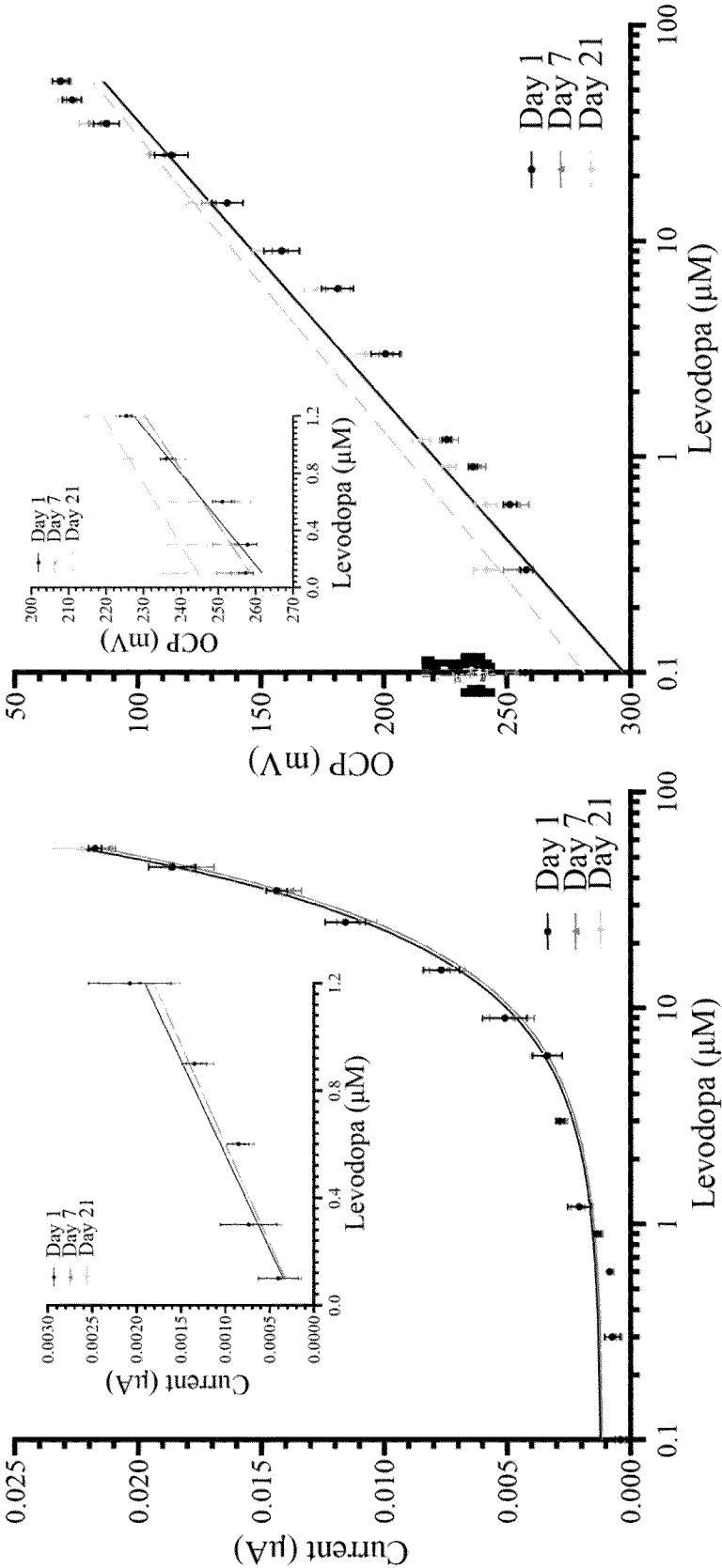


Fig. 34B

Fig. 34A

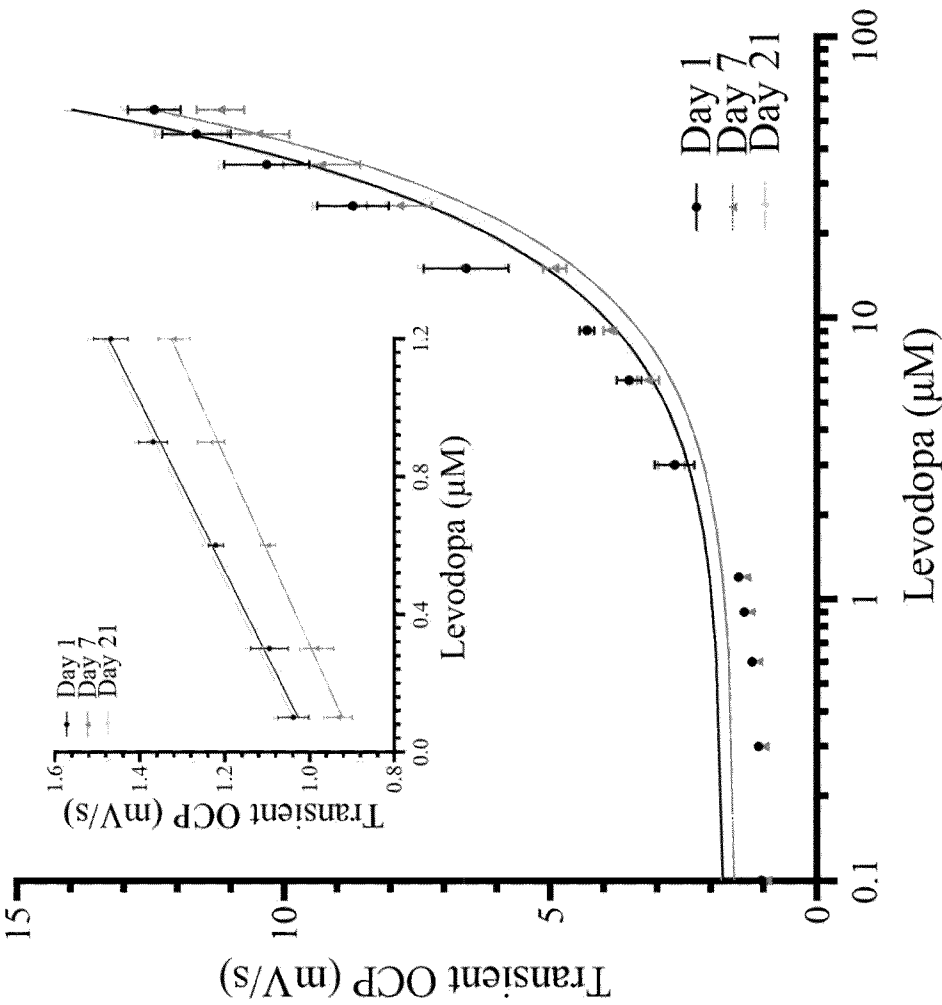


Fig. 34C

Fig. 35A

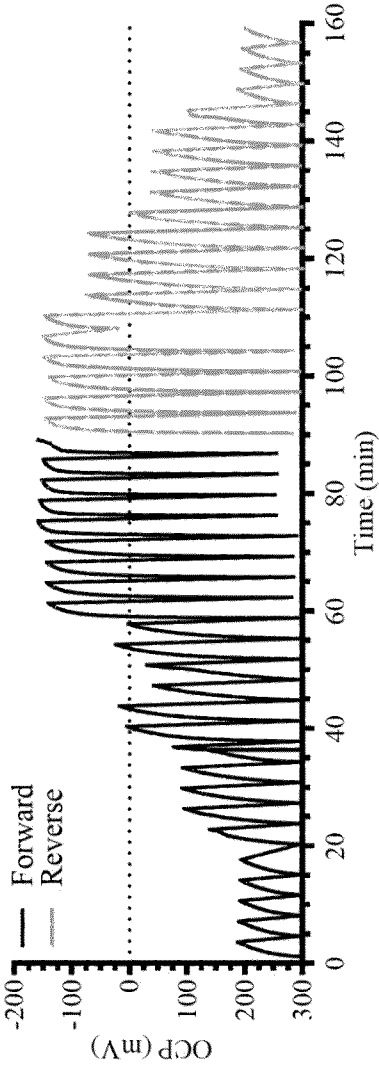


Fig. 35B

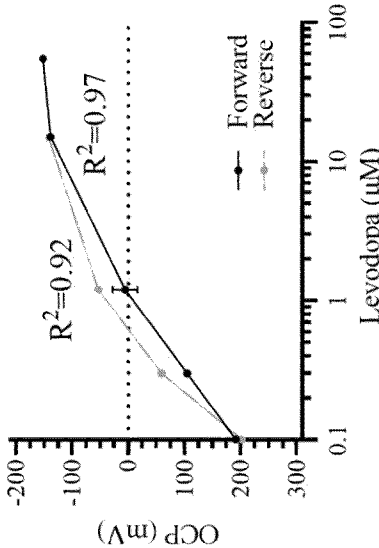


Fig. 35C

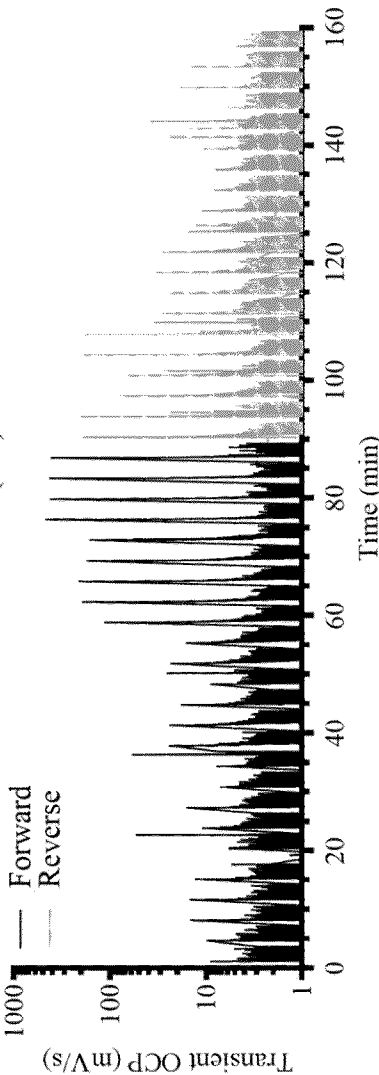
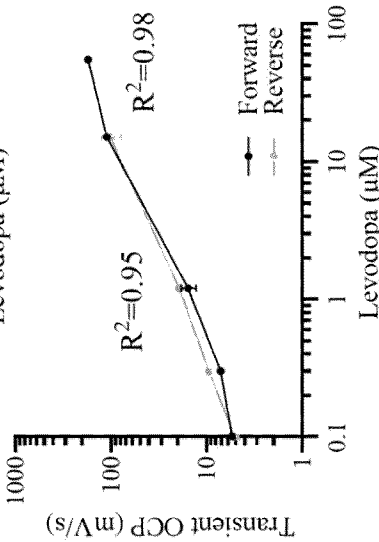


Fig. 35D



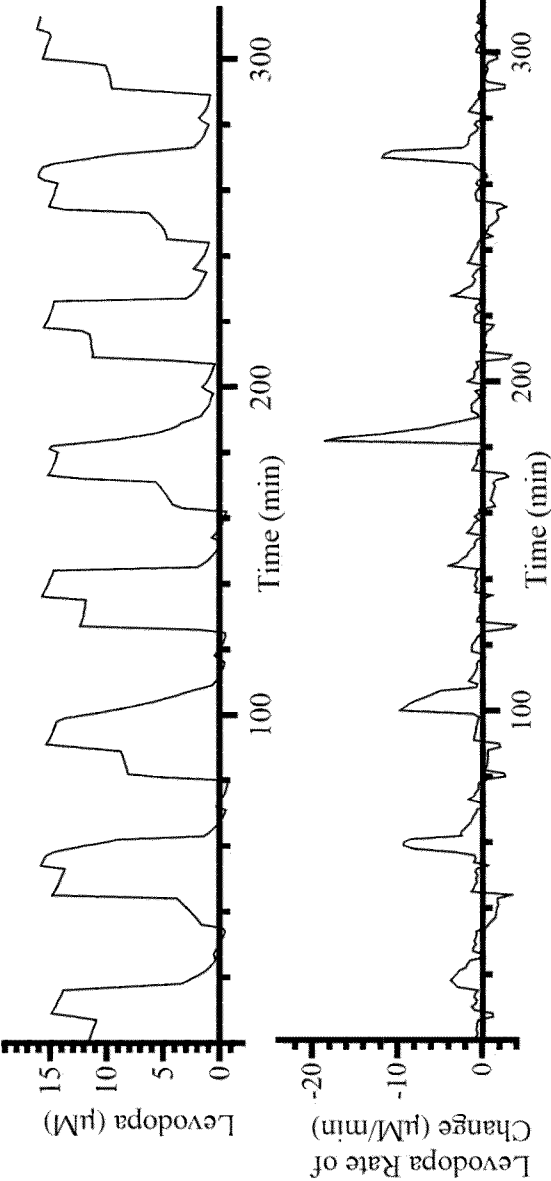


Fig. 36A

Fig. 36B

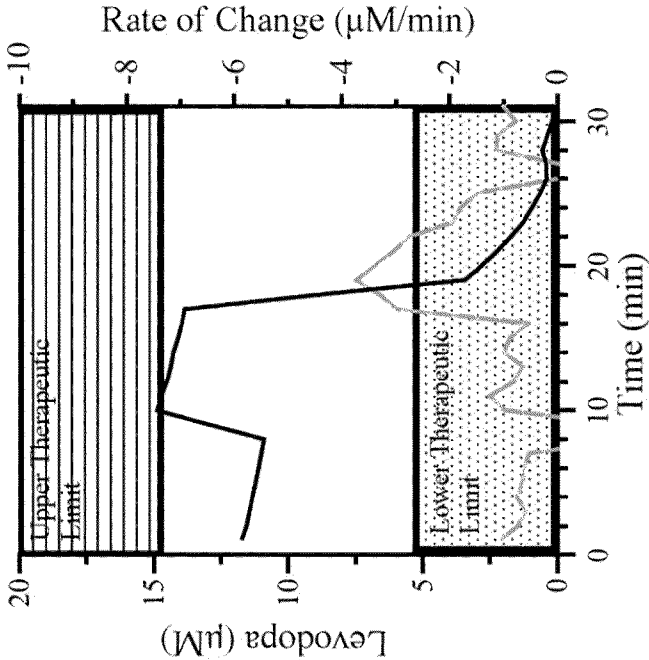


Fig. 36C

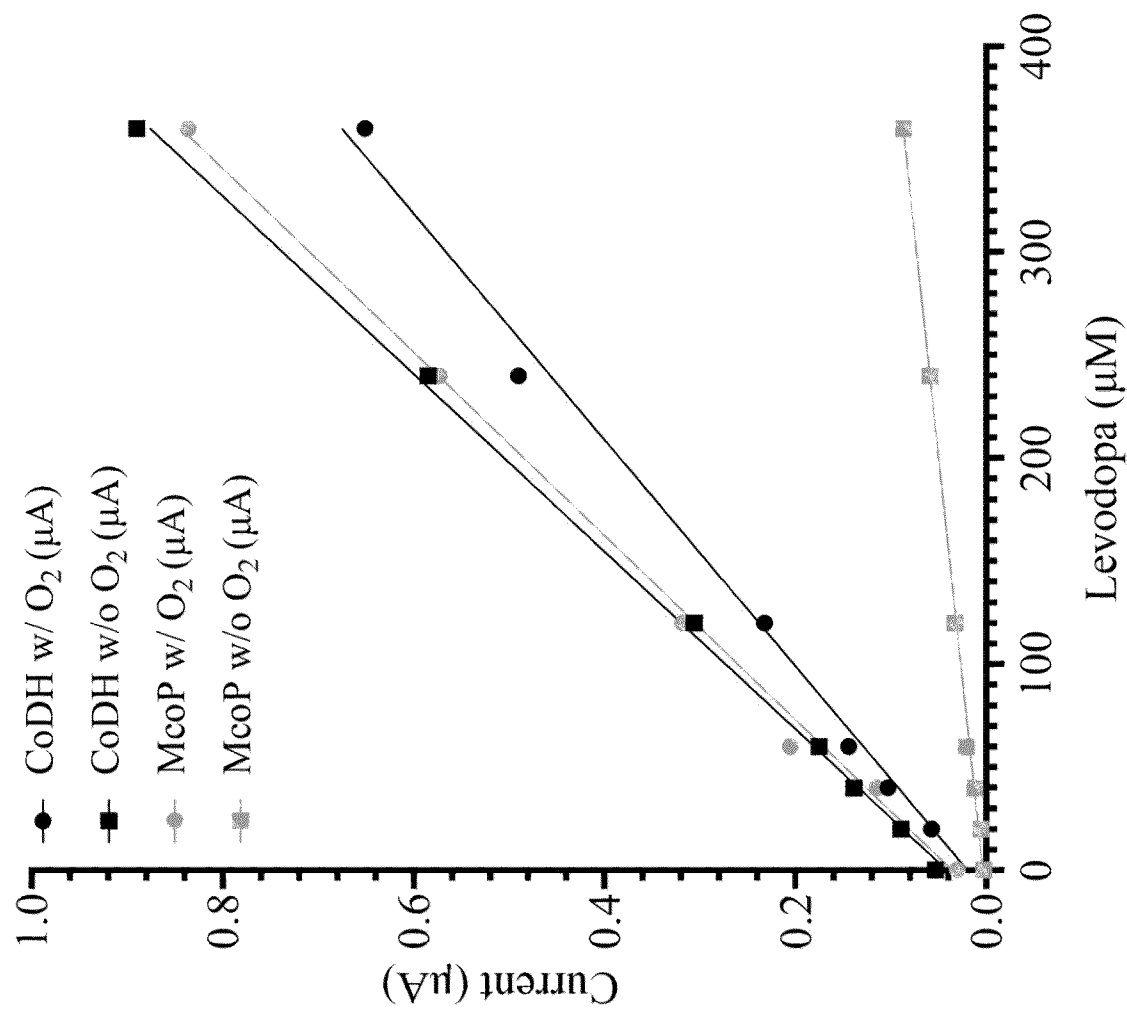
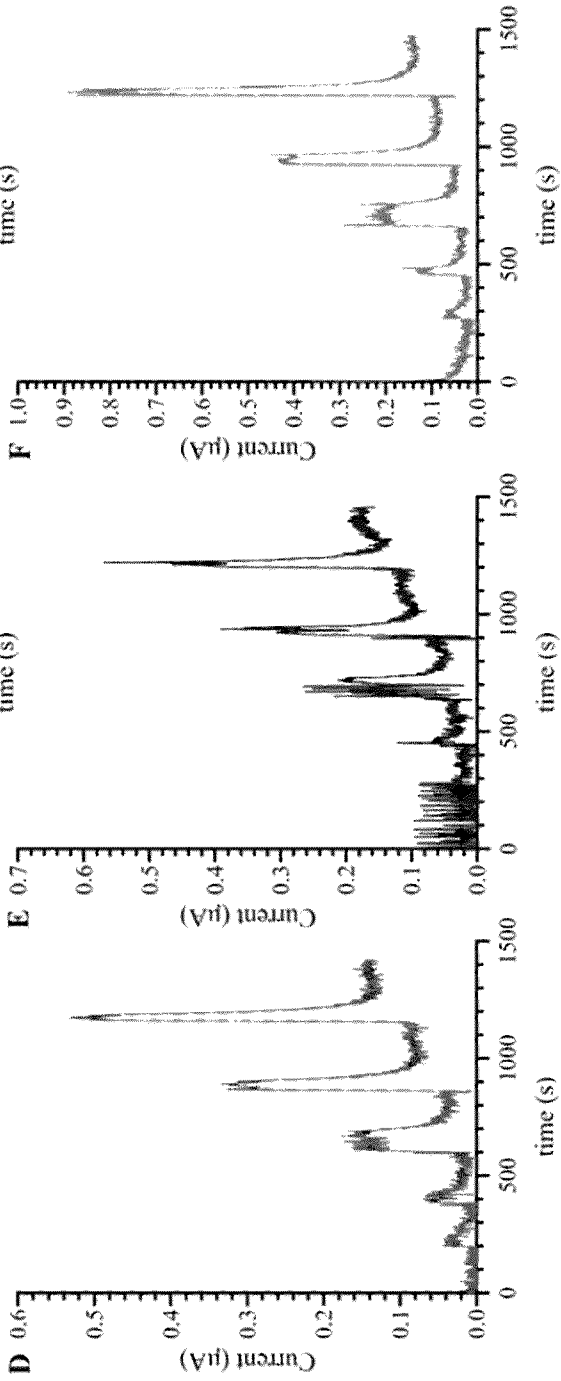
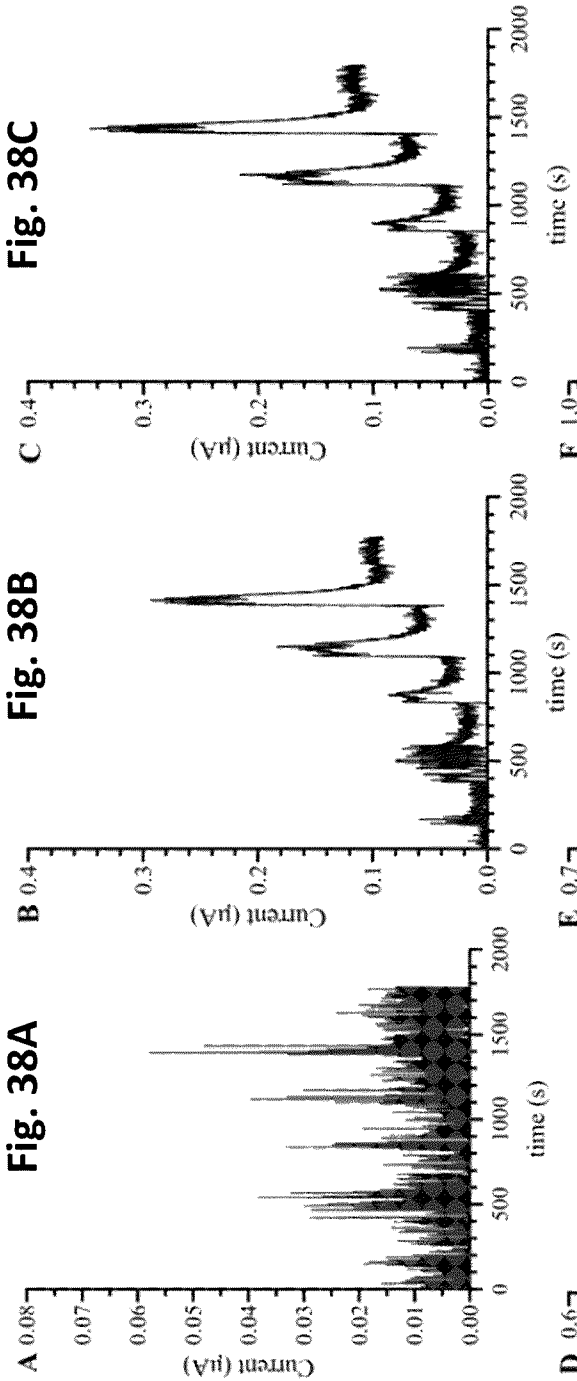


Fig. 37



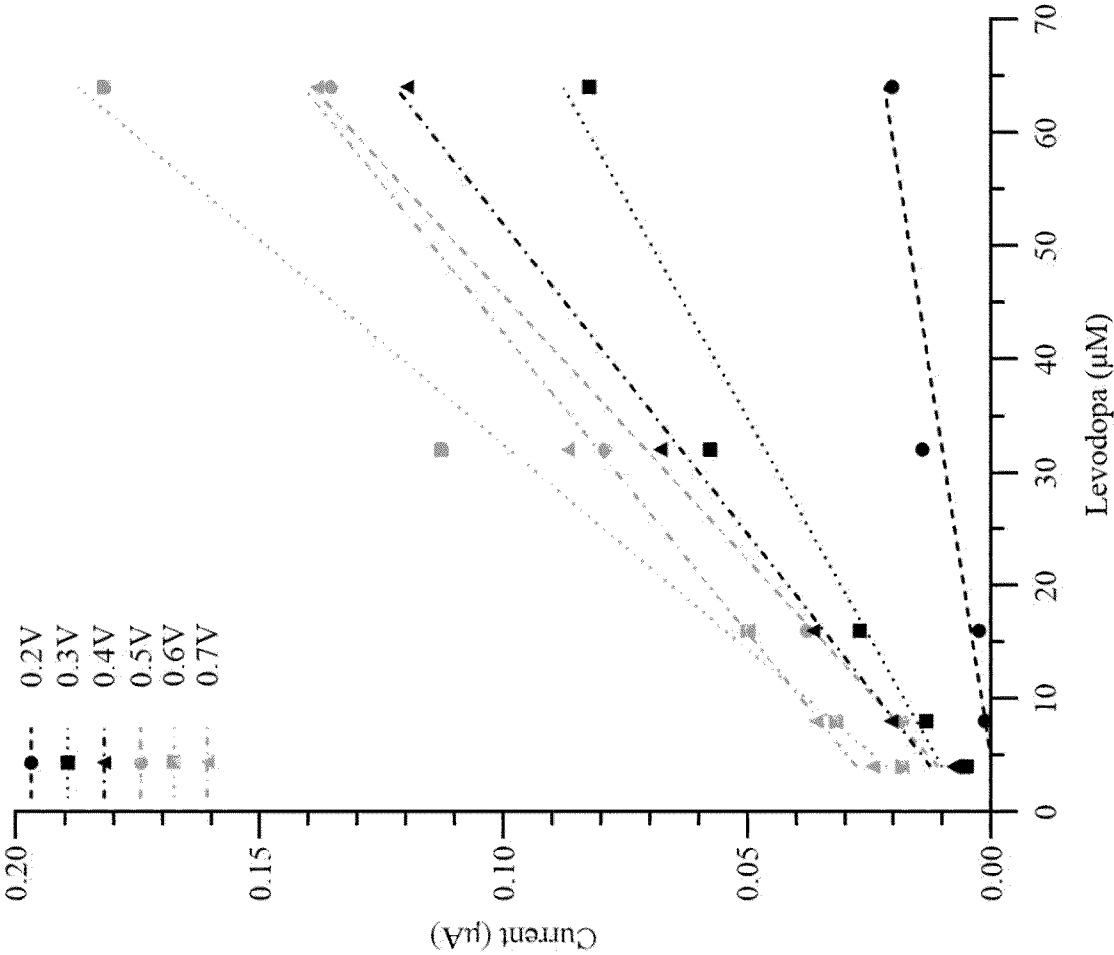


Fig. 38G

Fig. 39A

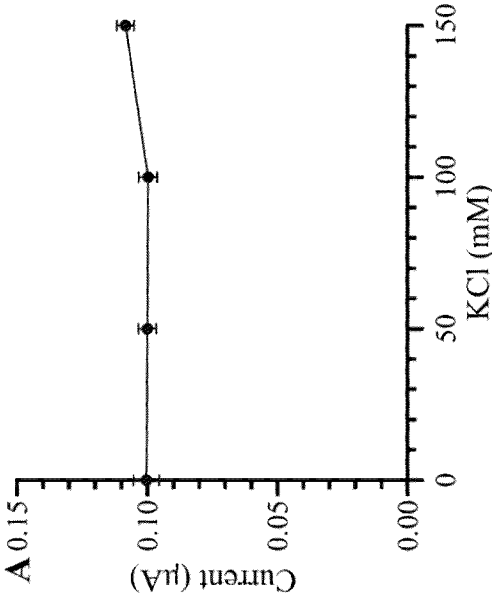


Fig. 39B

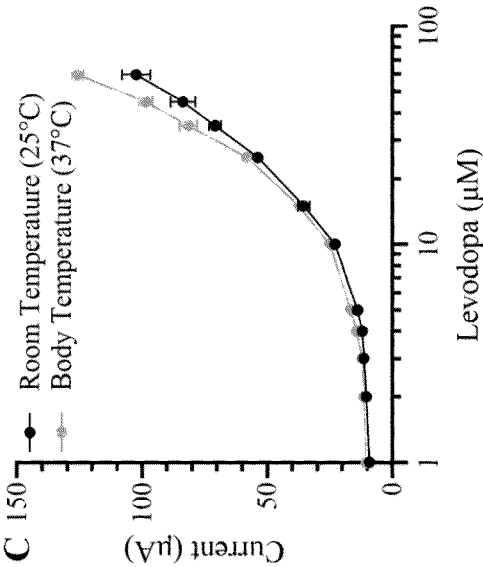
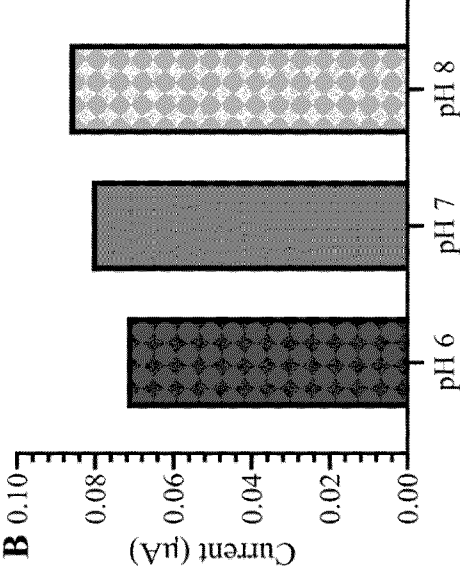


Fig. 39C

Fig. 39E

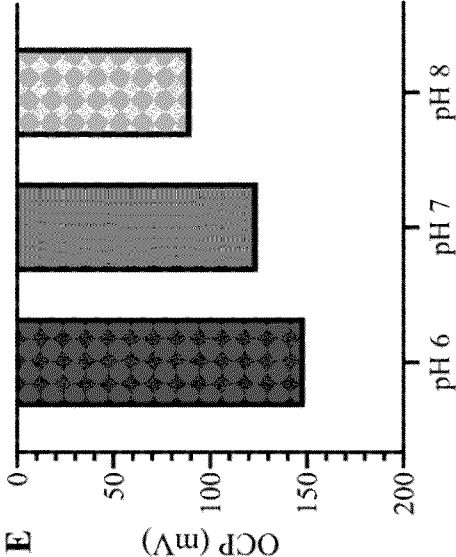


Fig. 39D

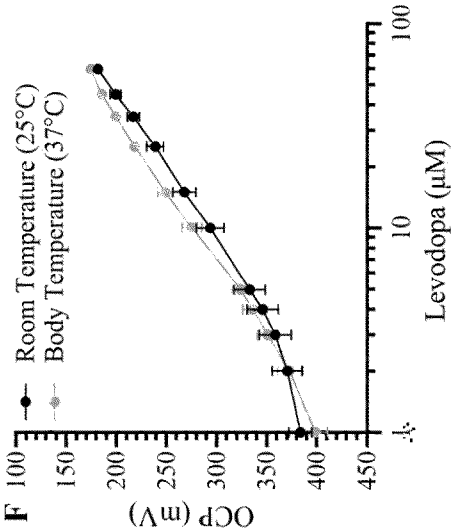
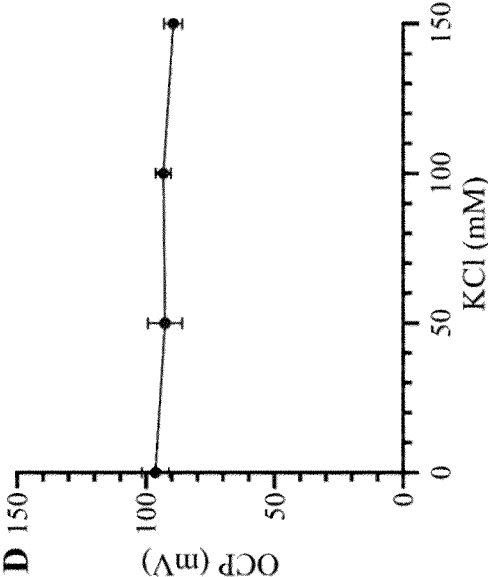


Fig. 39F

Fig. 39H

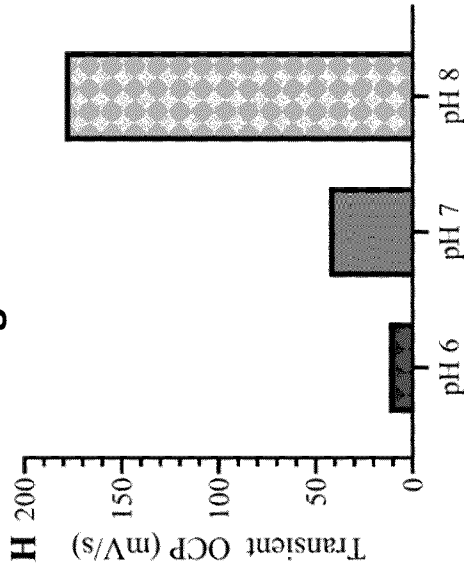


Fig. 39G

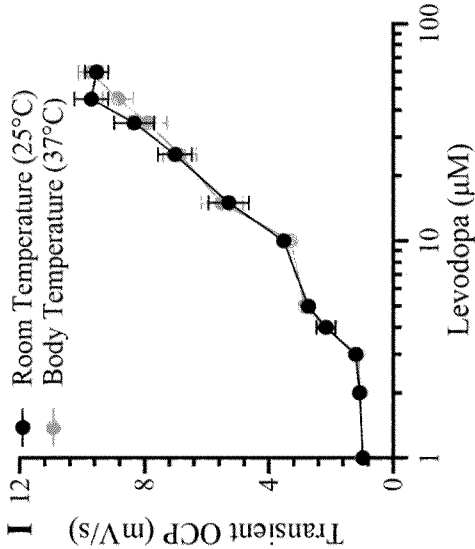
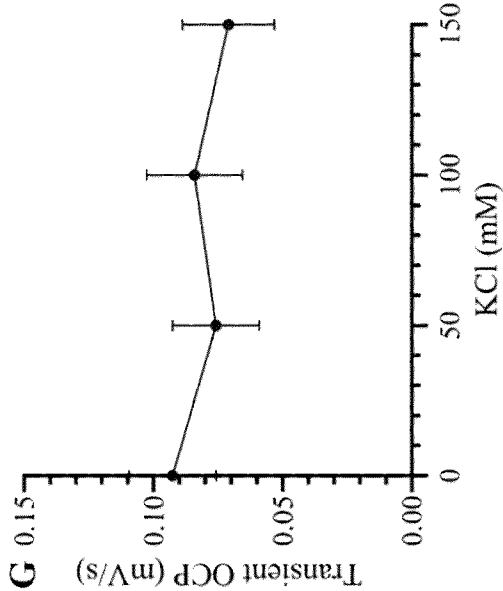


Fig. 39I

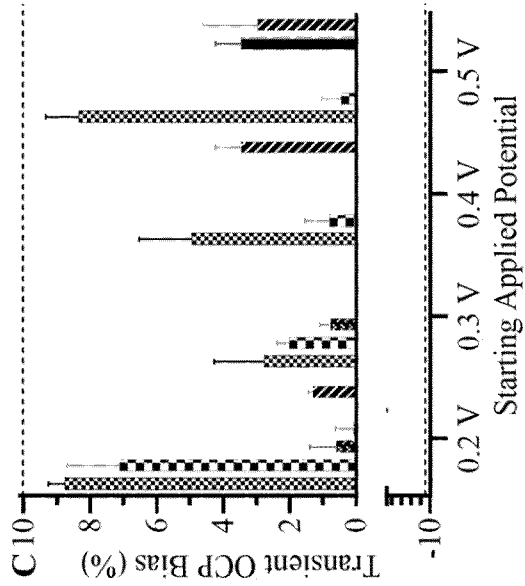
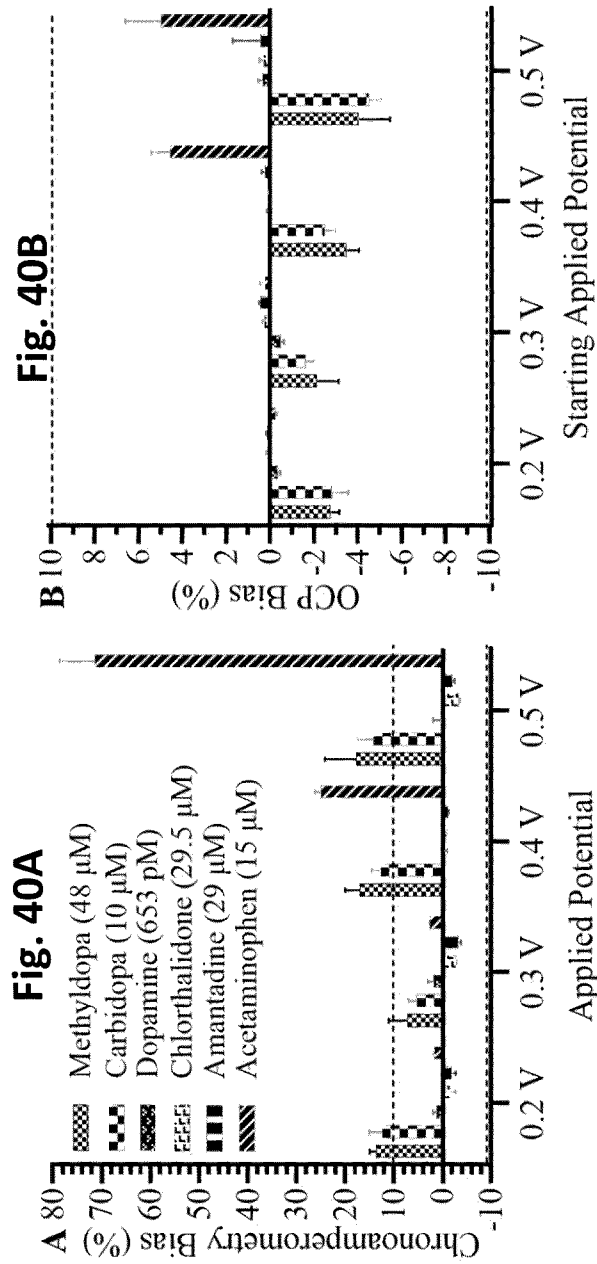


Fig. 41B

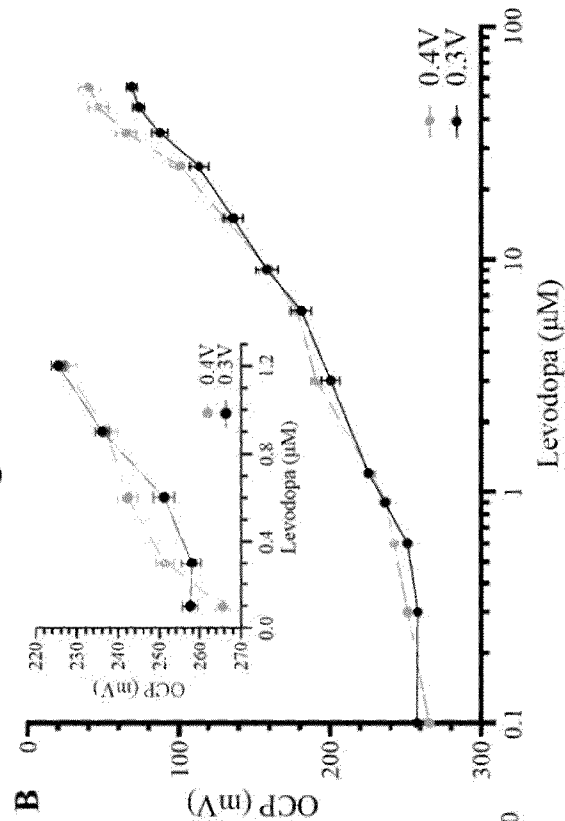


Fig. 41A

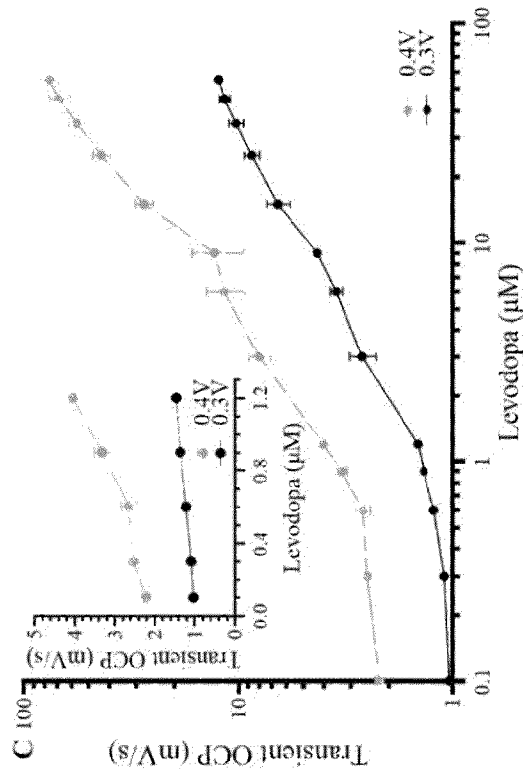
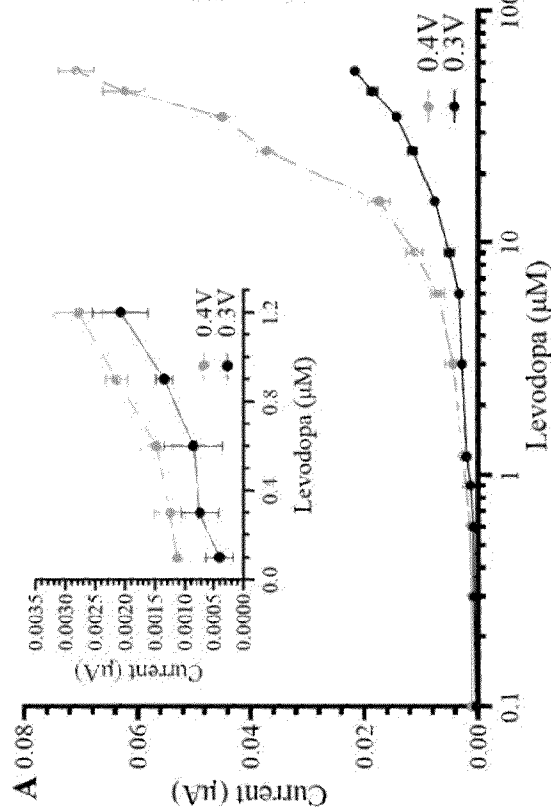


Fig. 41C

Fig. 41E

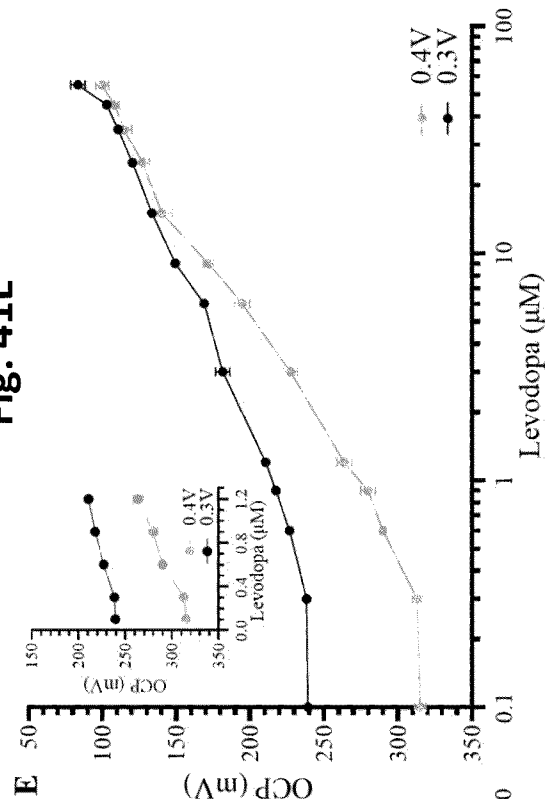


Fig. 41D

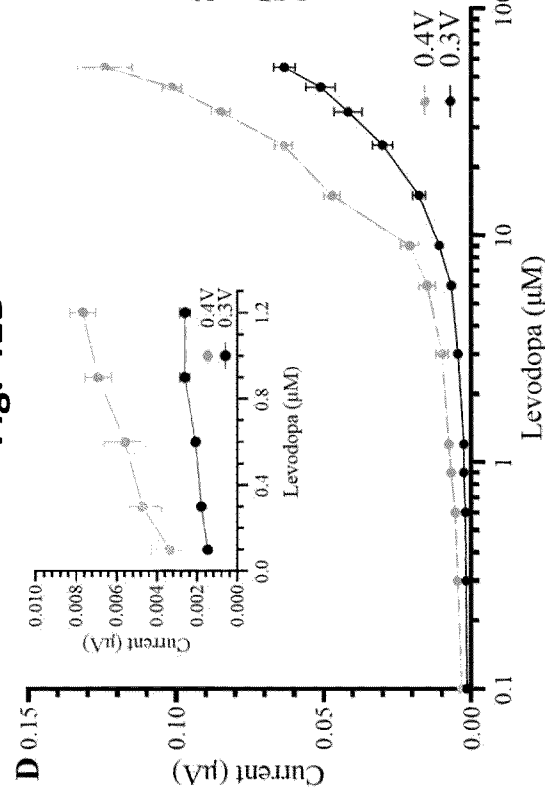


Fig. 41F

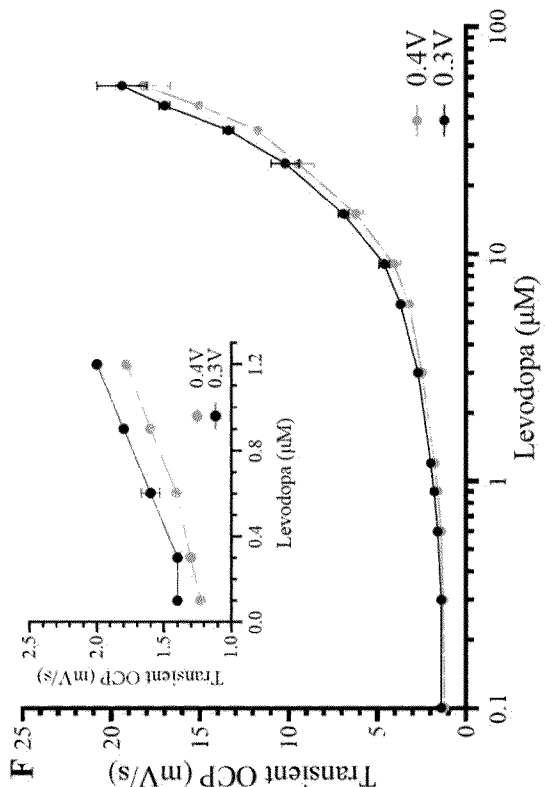


Fig. 42B

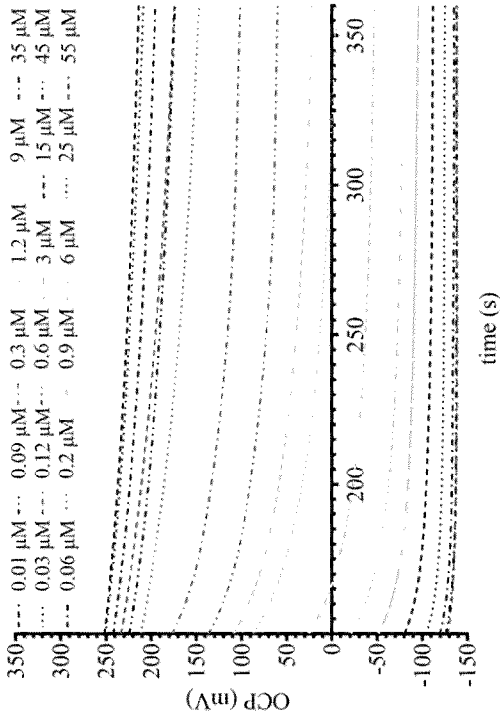


Fig. 42A

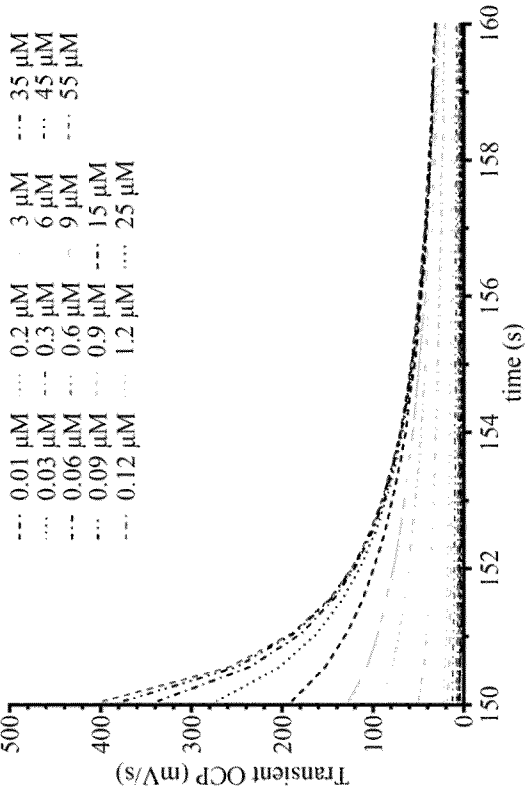
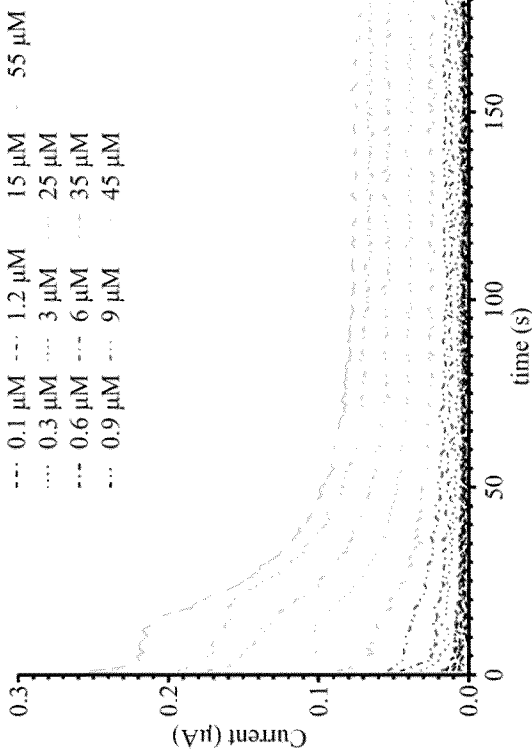


Fig. 42C

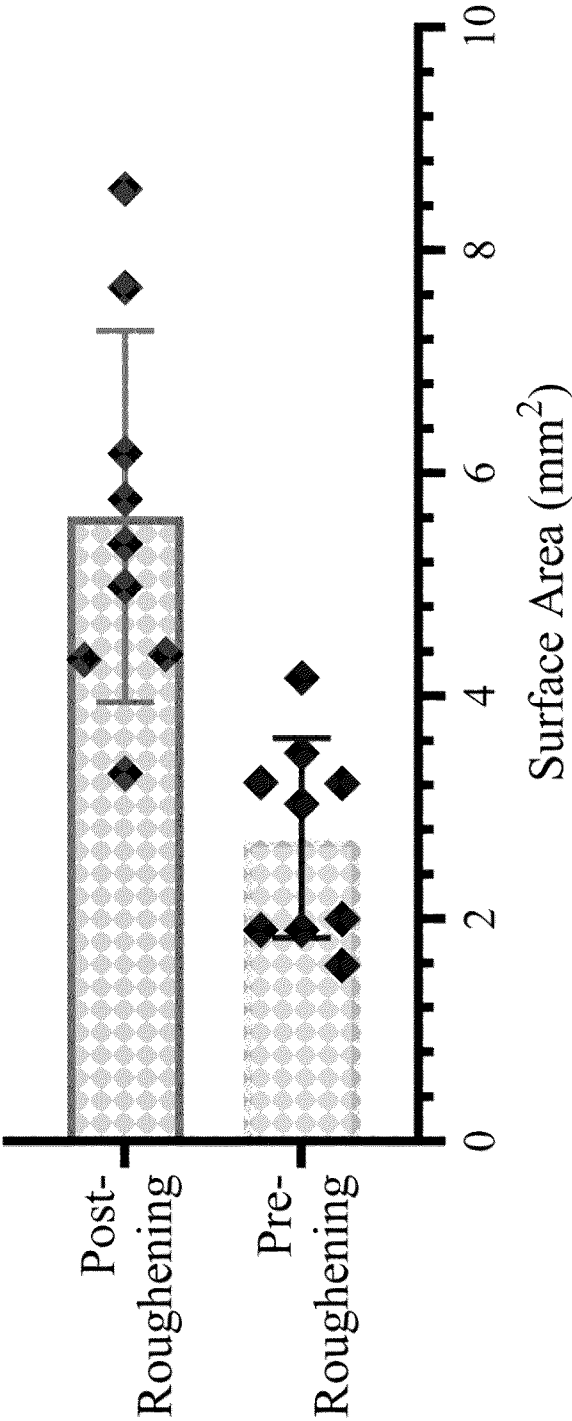


Fig. 43

Fig. 44B

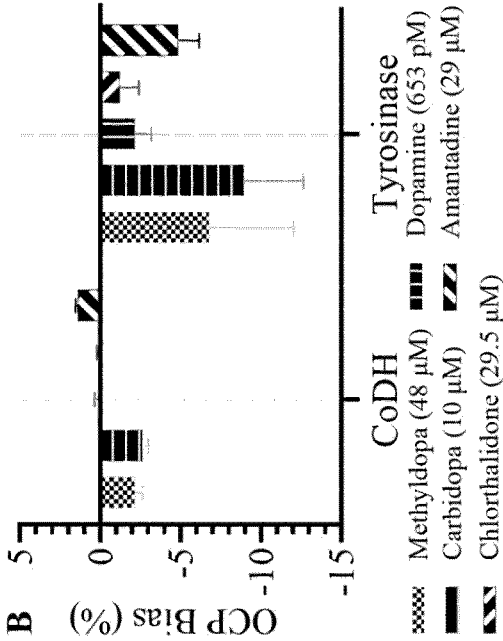


Fig. 44A

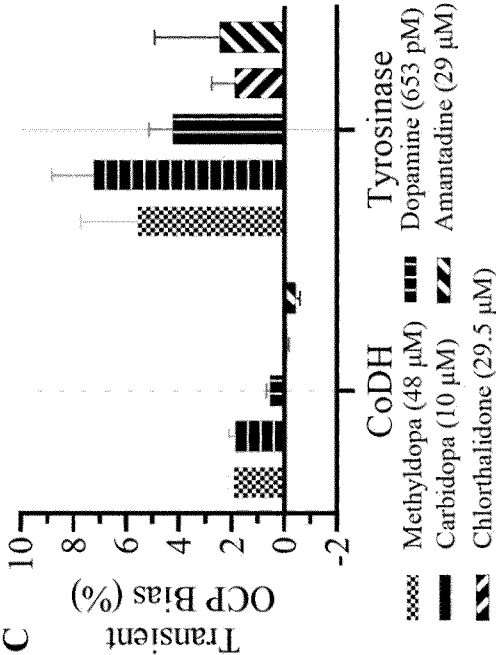
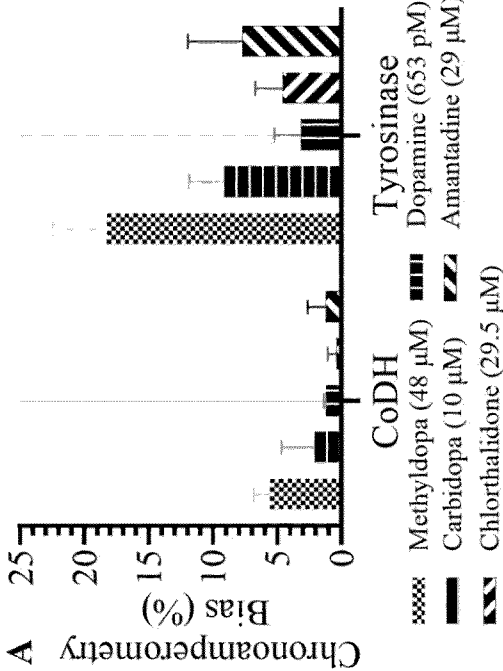


Fig. 44C

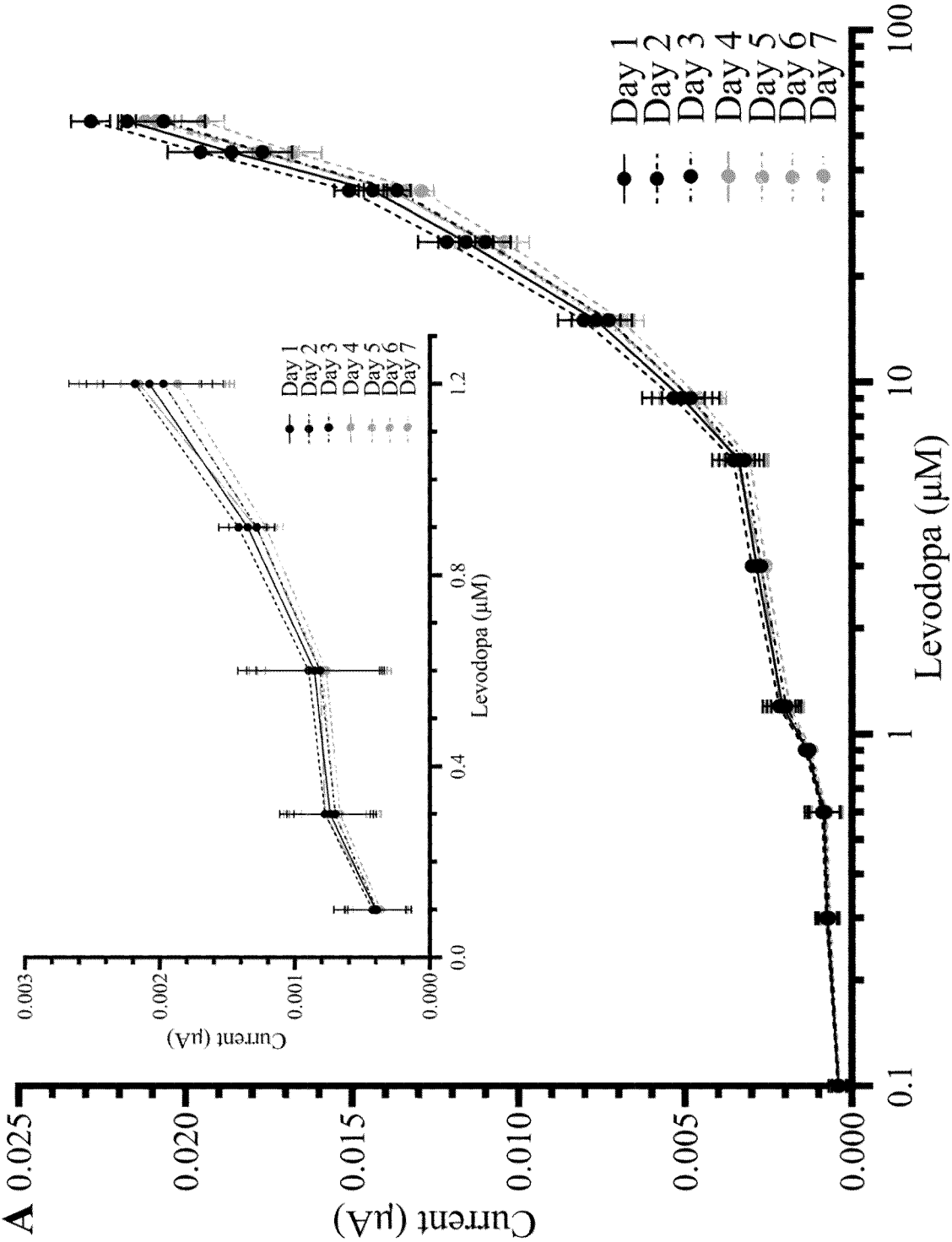


Fig. 45A

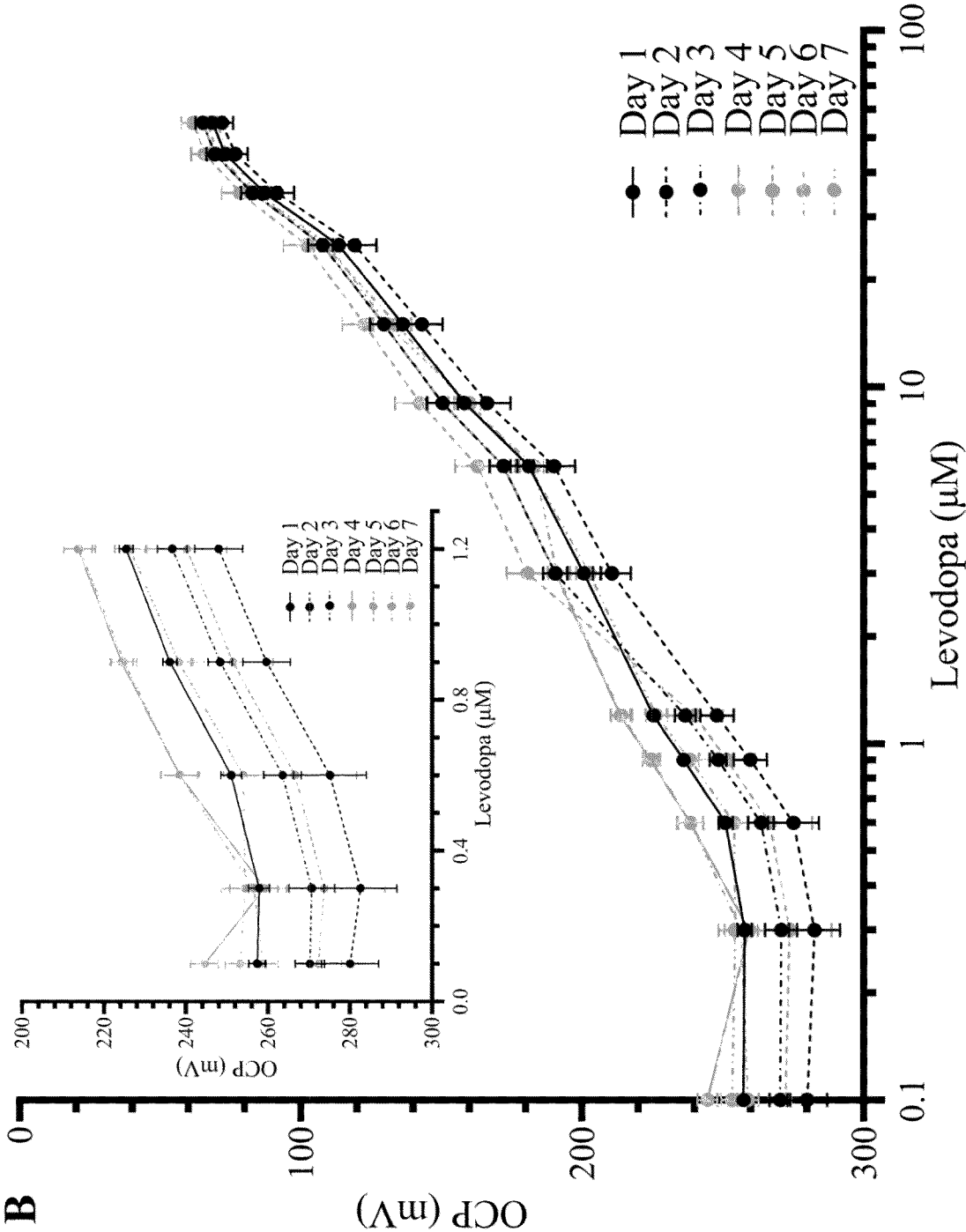


Fig. 45B

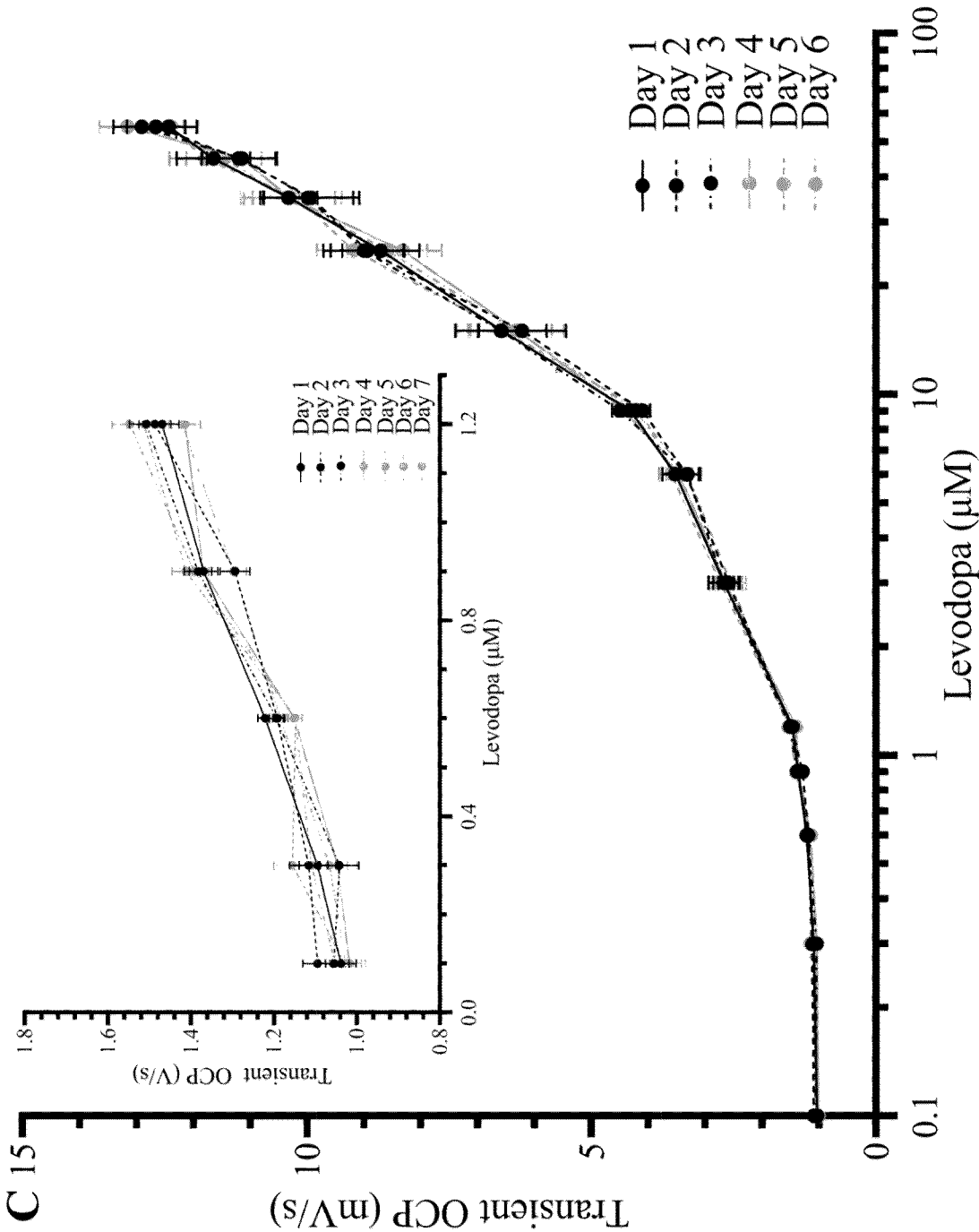


Fig. 45C

Configuration	Chronoamperometry			OCP		Transient OCP	
	LOD (μM)	Sensitivity ($\text{nA}/\mu\text{M}$)	LOD (μM)	Sensitivity ($\text{mV}/\mu\text{M}$)	LOD (μM)	Sensitivity ($\text{V}/\text{s}^* \mu\text{M}$)	
100 μM DSH-CoDH, 0.3 V applied	0.457	0.154	1.522	32.168	0.208	0.00064	
100 μM DSH-CoDH, 0.4 V applied	0.479	0.186	1.422	31.104	0.496	0.00143	
500 μM DSH-CoDH, 0.3 V applied	0.649	0.400	1.078	27.993	0.268	0.00033	
500 μM DSH-CoDH, 0.4 V applied	0.715	0.185	3.822	48.901	0.327	0.00097	

Fig. 46

Fig. 47A

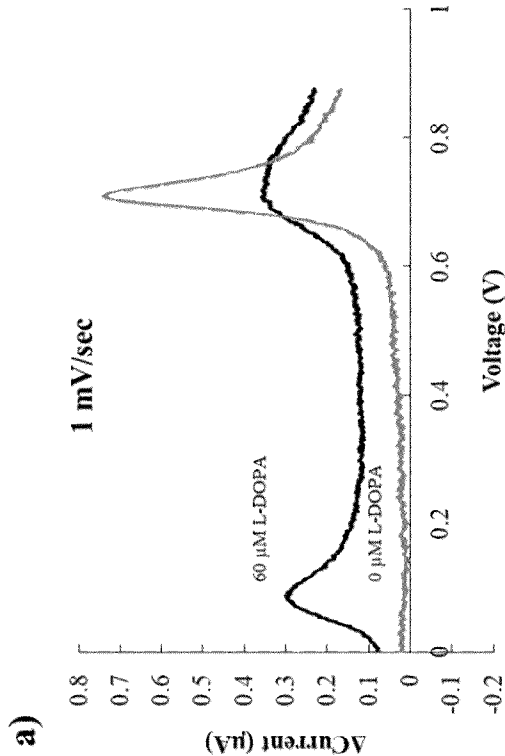
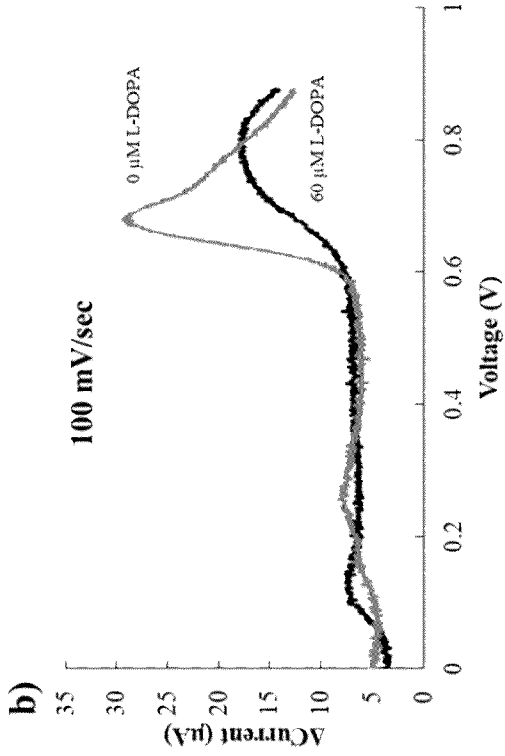


Fig. 47B



c)

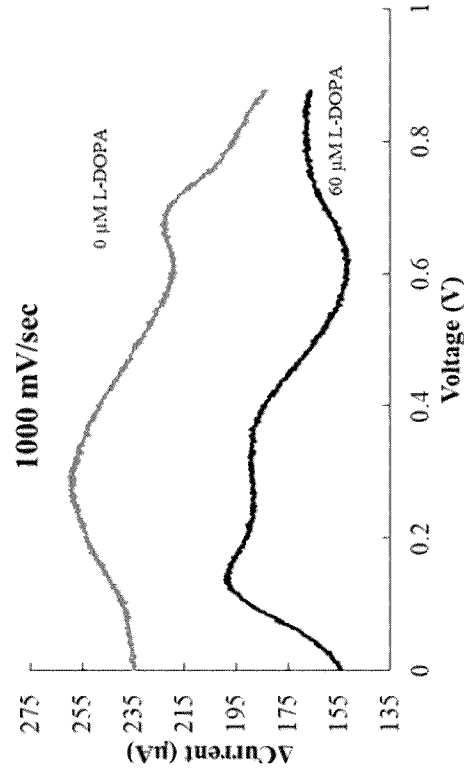


Fig. 47C

Fig. 47E

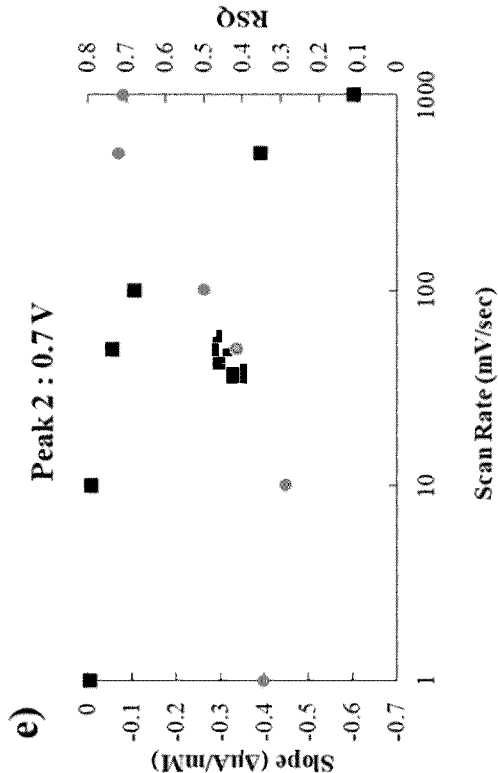


Fig. 47D

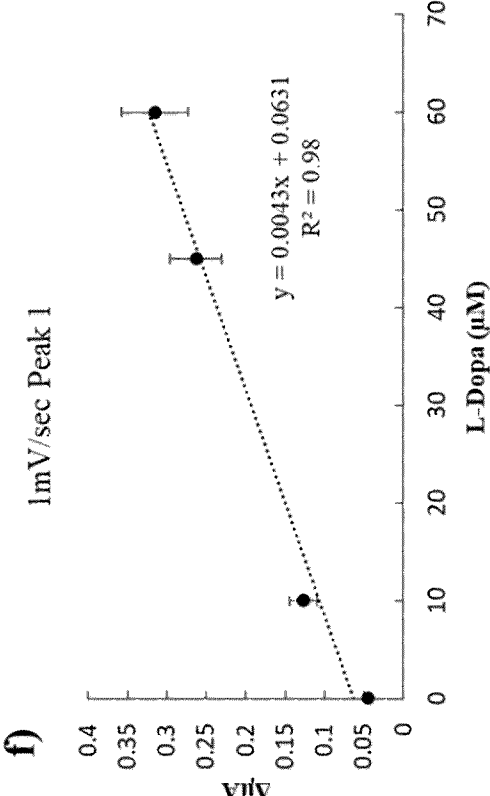
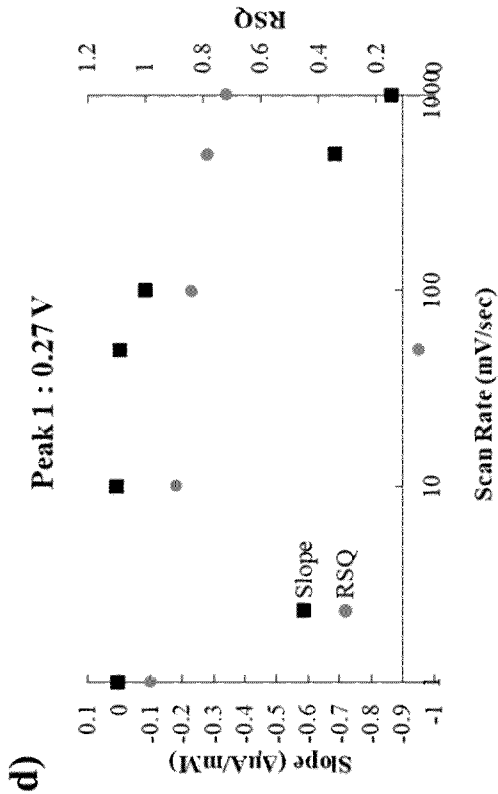


Fig. 47F

Fig. 48A

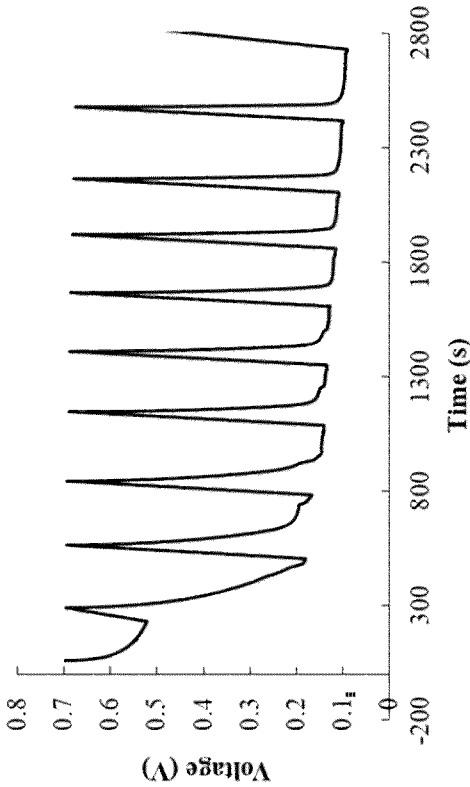


Fig. 48B

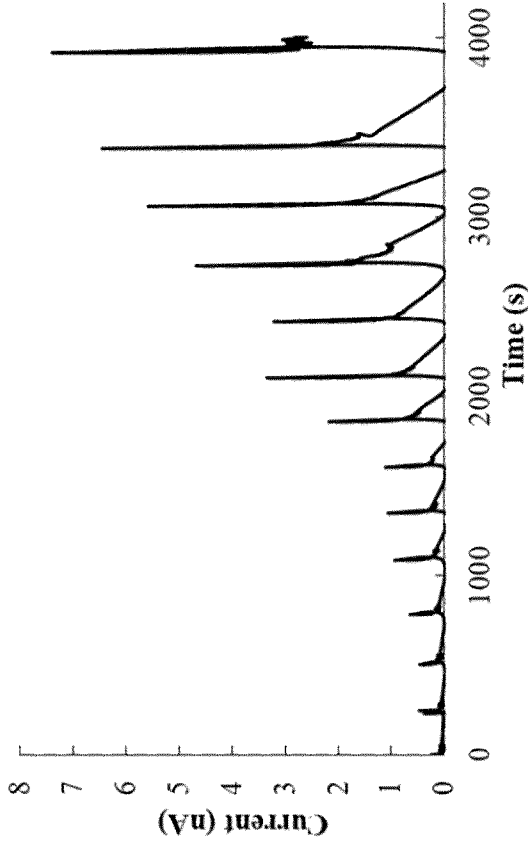
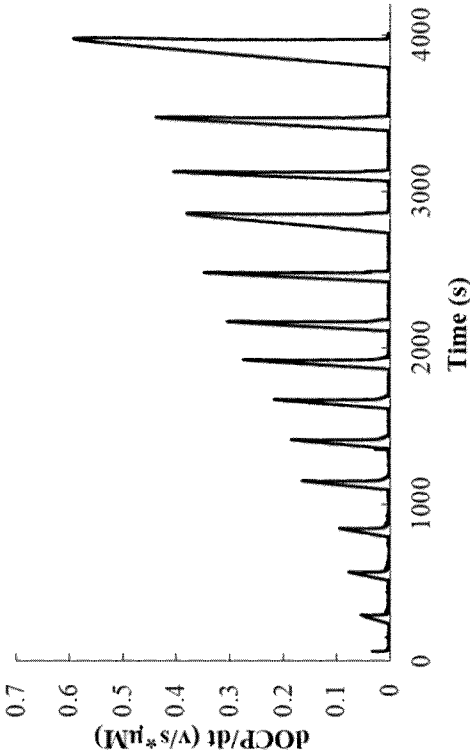


Fig. 48C

Fig. 48E

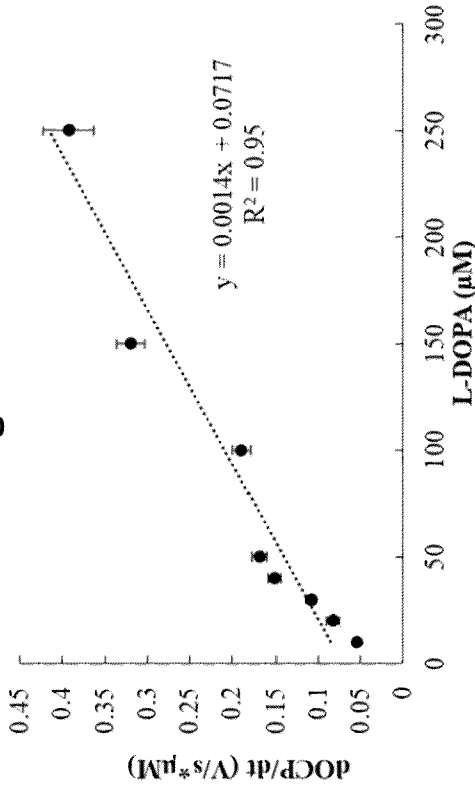


Fig. 48D

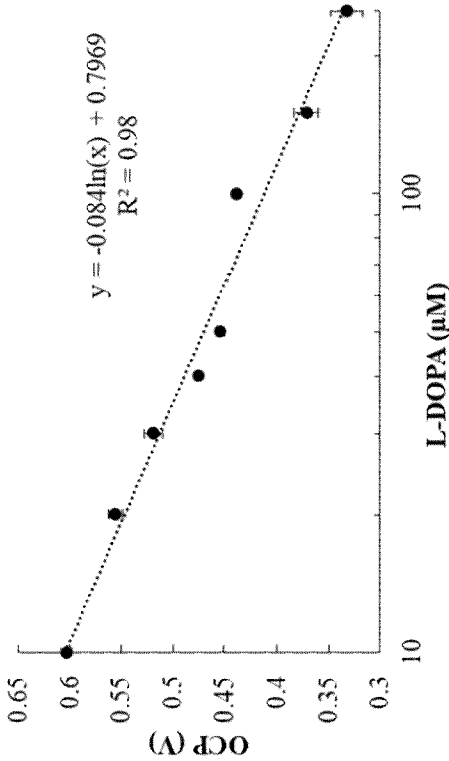
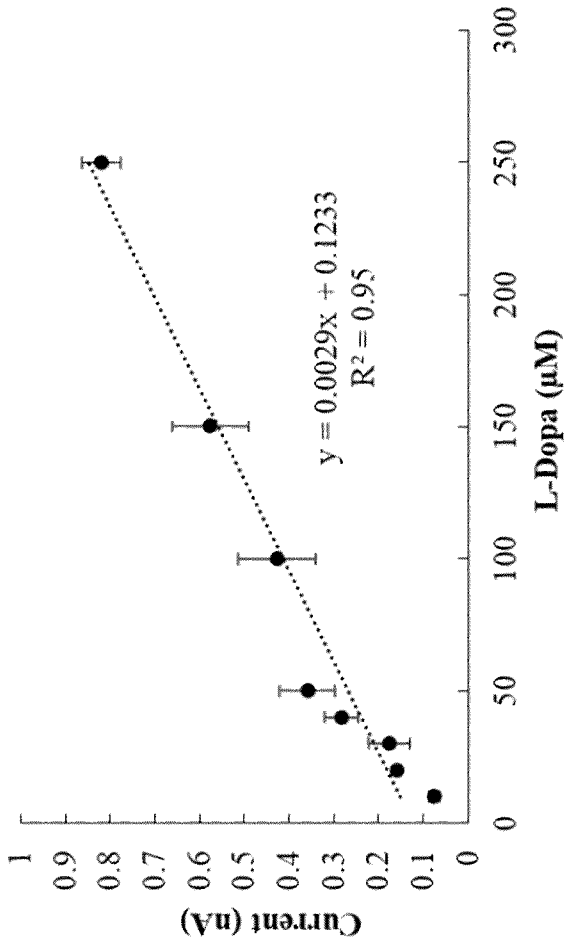


Fig. 48F



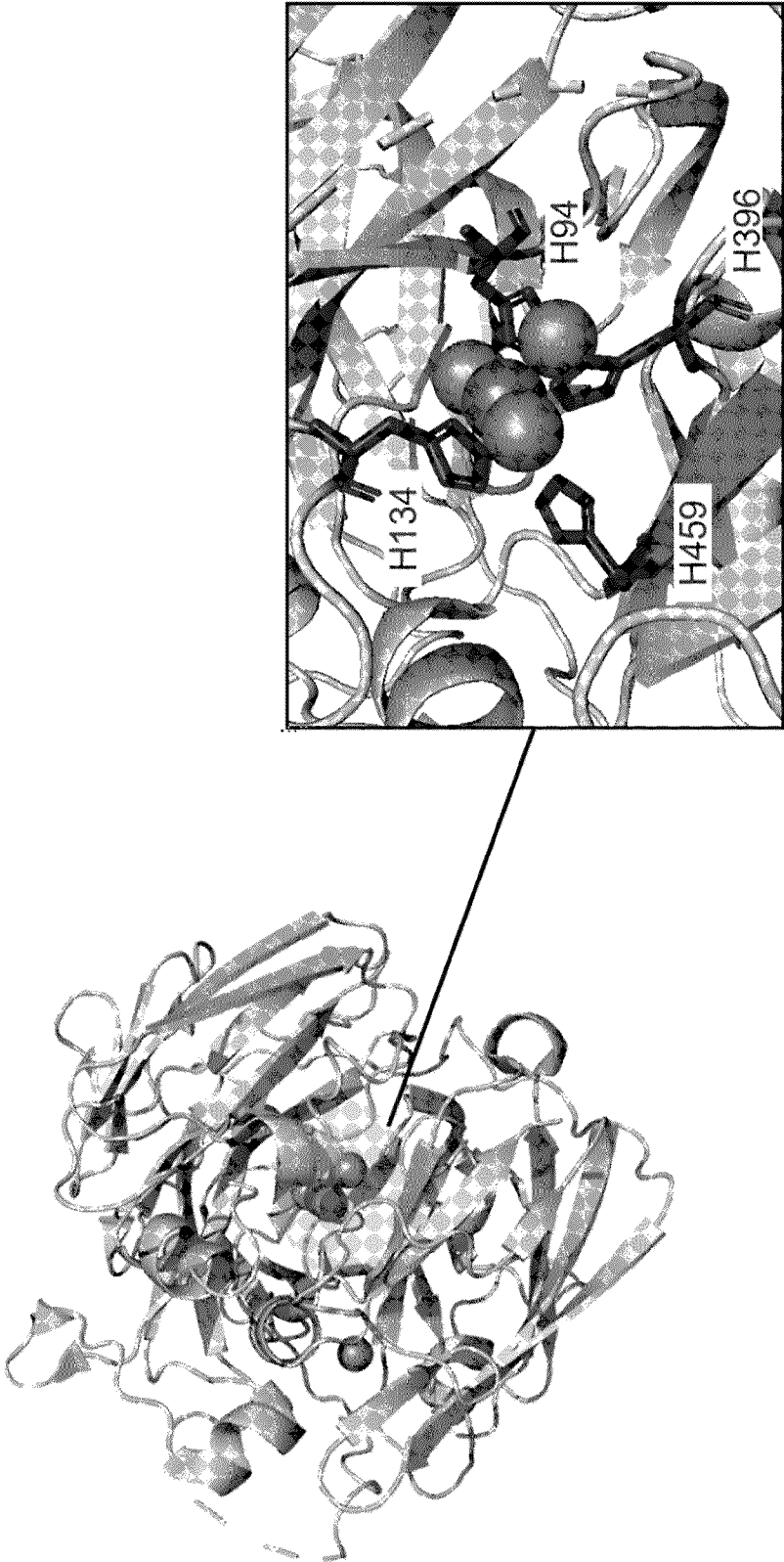


Fig. 49

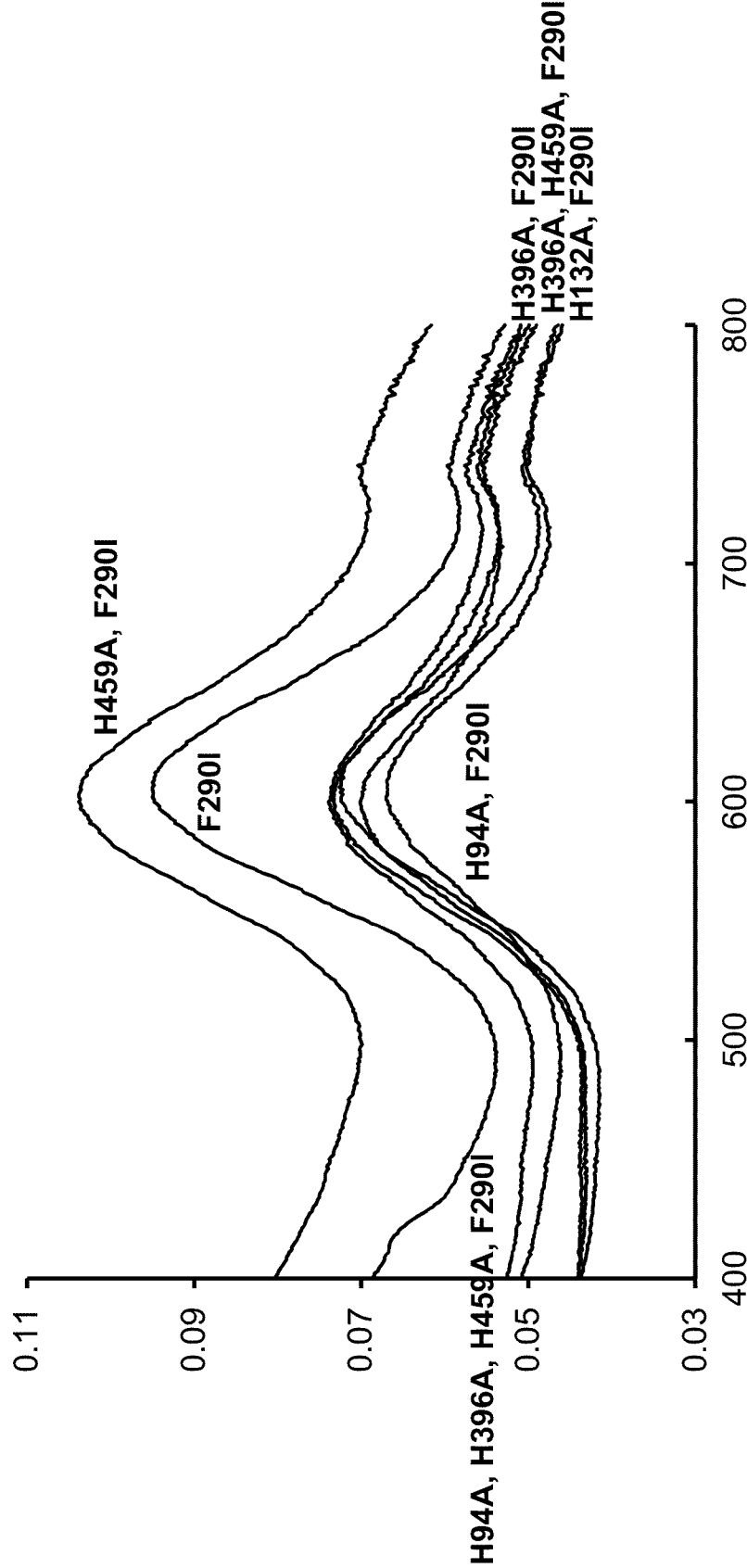


Fig. 50

McoP
CotA
CueO
TTMCO
BpLac
BilLac
SclLac
SllLac
MvBOD
Clustal Co

10 20 30 40 50 60 70 80 90 100

-----SAP VPPLIKEATY IEATASGYMA
-----YVEVT MEETHQLHR DLPPTRLWGY
-----NRIQ LTIGACQSTF GSKTATTWGY
G-----LLEVR LKAAPTPTV AGREARLWTY
-----YVEIA MEEVEFLKVRH DLPPTKLWTY
-----YVEVT MKEFOOQLHR DLPPTRLFGY
-----ETEIA LRPAWVRLHP QLPPTIMWGY
-----ETEIA LRPTWVRLHP QLPPTIMWGY
MFKHTLGAAA LSLLFNSNAV QASVPETSP ATGHLKRVQ QISEQYPMET VLPPIPEVKQ ERLIVTNEVN GQEIWYVEVE IKPFTHQVYP DLGSADLVGY

McoP
CotA
CueO
TTMCO
BpLac
BilLac
SllLac
MvBOD
Clustal Co

110 120 130 140 150 160 170 180 190 200

EGVLN-PTII LREGQVDMT LKN
NGLEPGPTIE VKRNEYVVK WMNLPSSTHE LPIDHTIHS DS-
NGNLLGPAVK LQKGAVTVD IYN-
GGSPGPTLR VRPGDTPVRLK LEN-
NGSLPGPTIH ANRNEKVVK WMNKLPLKHE LPVDHTIHEG
NGVSPGPTIE VQKHEKVAVK WINKLPDRHE LPVDHTLHDD G-
DQVPGPTIE VRRQVRVIA WTNRIKPKDSE YPVTSEVVEL RTDGRPQSTT EPGREGVEFN KDVAALPAWS VTEHGAQTG GGNDGWADNA VG-
GGSPGPTIE VRRQVRVIA WTNRIKPKGSE YPVTAVEVPA AN-PNFT EPGRGVPPPI ADVAALPAWT VTEHGAQTG GGNDGWADNA VG-
DGMSPGPTFQ VPRGVEVVR FIN-
* : *

McoP
CotA
CueO
TTMCO
BpLac
BilLac
SclLac
SllLac
MvBOD
Clustal Co

210 220 230 240 250 260 270 280 290 300

PGESYNSFD VNRAGTYLY
YKREVVHYYP NQQRGAILWY
PGGRSVTLN VDQPAATCFW
RETWSYVFTV PQDLAGTFWY
FFEREVYYP NHQOACTLWY
FFEREVYYP NEQDATLWY
FGDQQLSEXP NDHQAVQWY
YGDQQLSEXP NDHQAVQWY
EGSKFDYYYP NRQSARTLWY
* : *

○ The amino acid residues substituted in McoP
□ Involvement in T2/T3 copper coordination

Fig. 51



Fig. 51

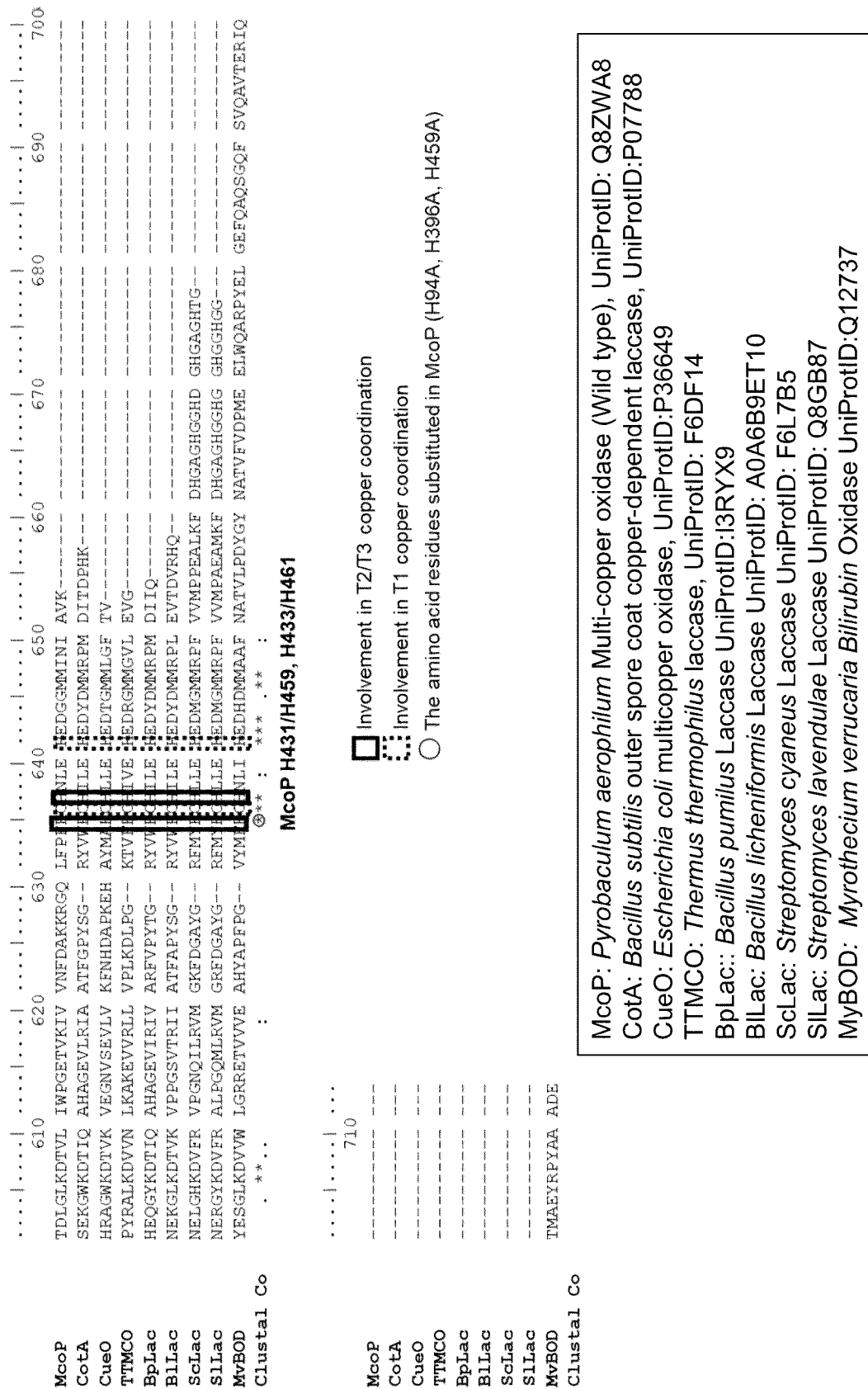
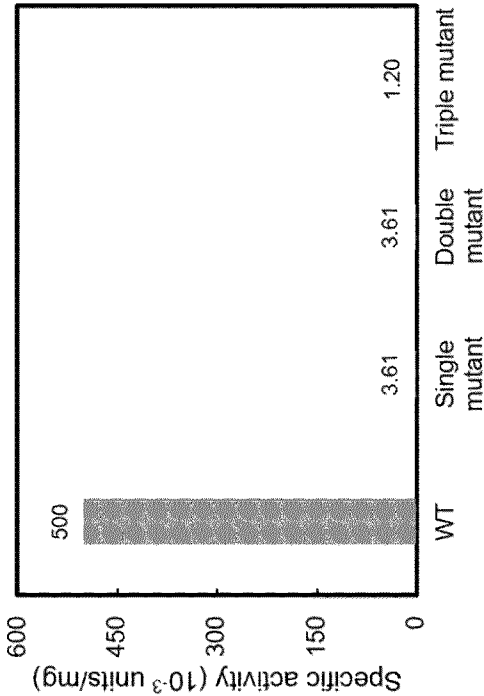


Fig. 51

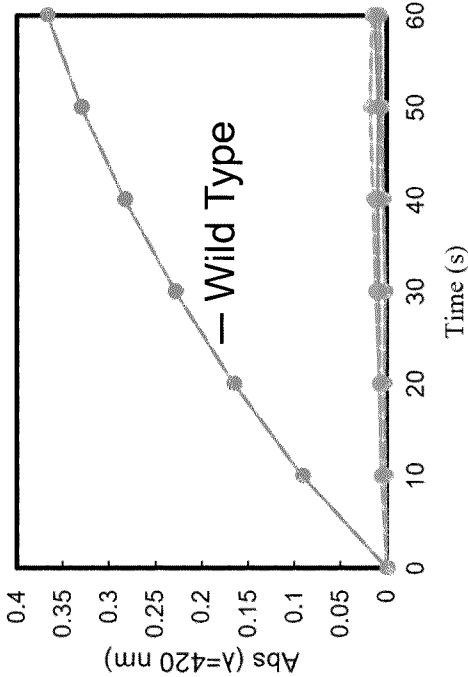
Fig. 52A



- Wild Type
- Single mutant (H448A)
- Double mutant (H448A, H499A)
- Triple mutant (H103A, H448A, H498A)

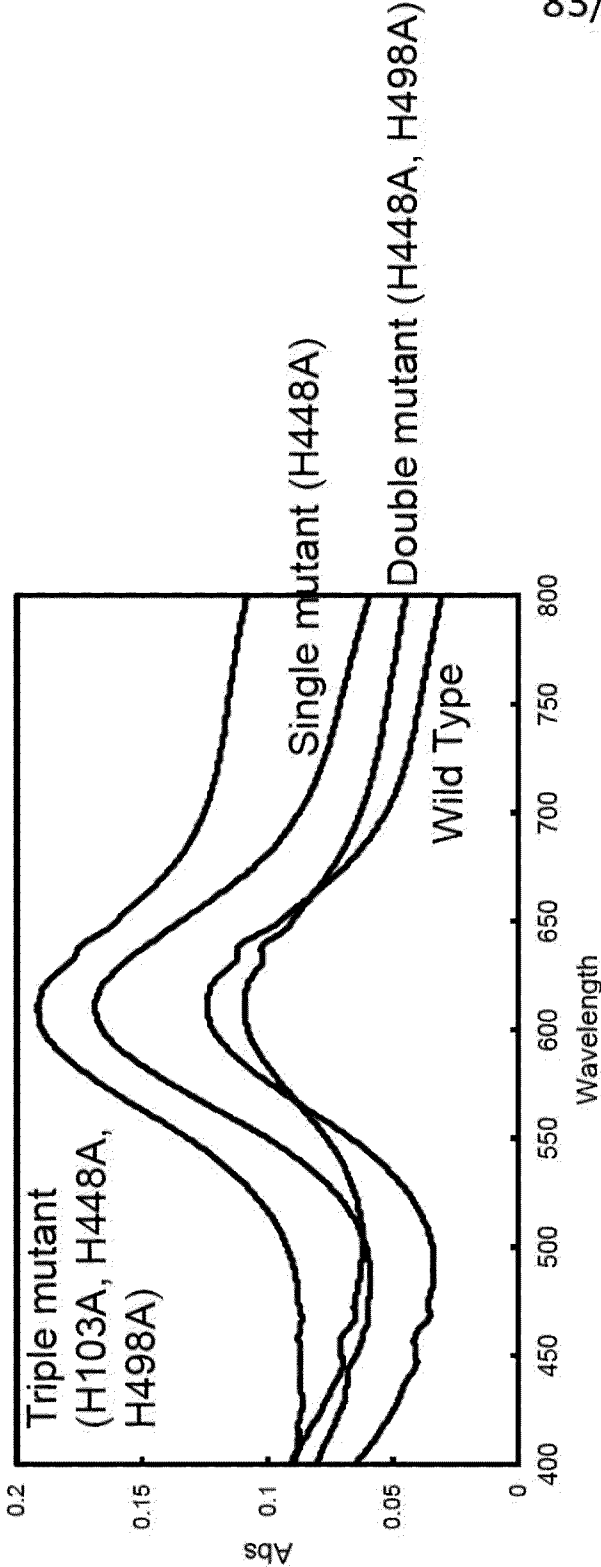
Fig. 52B

Absorbance change at 420 nm for 1 min by MCO activity



Measurement condition of MCO activity			
200 mM	Britton-Robinson buffer (pH 3.5)	500 μl	
	Enzyme(2 mg/ml)	50 μl	
	RO Water	400 μl	
	Incubate(2 min, 37°C)	950 μl	
20 mM	2-2'-Azino-bis(3-ethylbenzoline-6-sulfonic acid) diammonium salt	50 μl	
(ABTS) _ε = 36 mM ⁻¹ cm ⁻¹			(λ = 420 nm)

Fig. 52C



(Protein concentration: 2 mg/ml)

Fig. 54

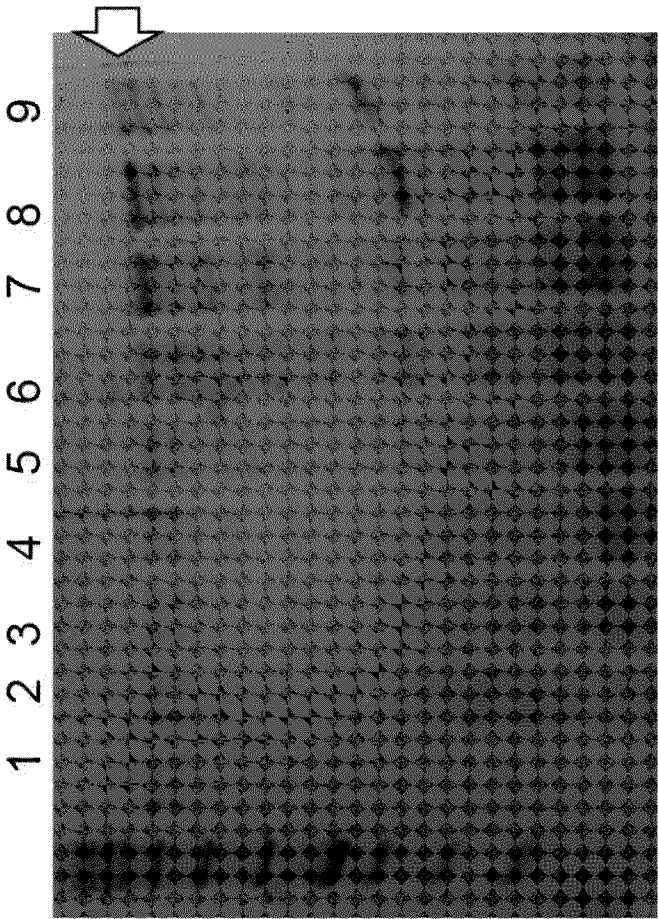


Fig. 53

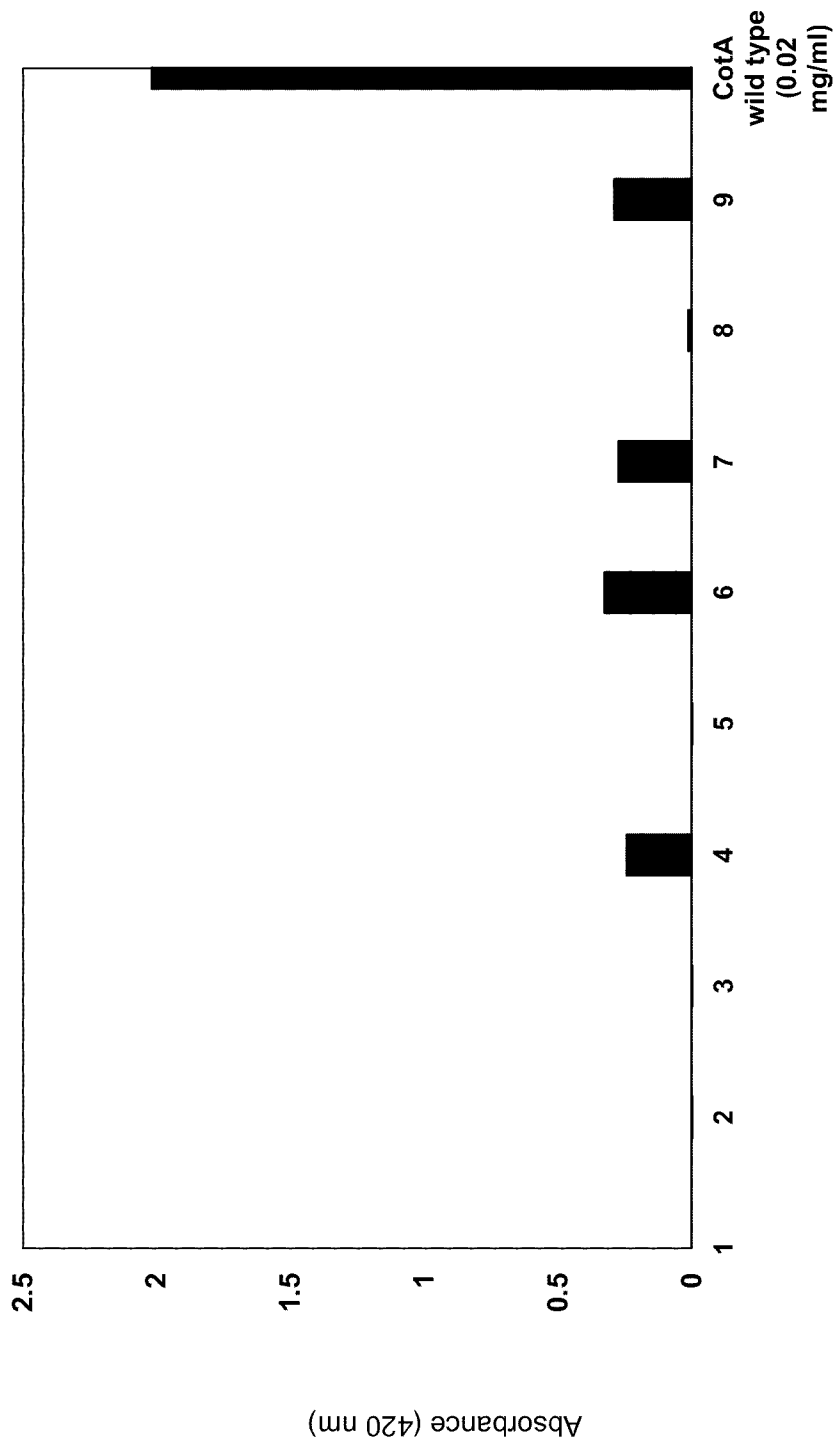


Fig. 54

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/031682

A. CLASSIFICATION OF SUBJECT MATTER C12N 9/02(2006.01)i; G01N 27/327(2006.01)i; C12Q 1/32(2006.01)i; C12Q 1/00(2006.01)i; H01M 8/16(2006.01)i; H01M 4/90(2006.01)i 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) C12N 9/02(2006.01); A23L 5/20(2016.01) Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Korean utility models and applications for utility models Japanese utility models and applications for utility models Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) eKOMPASS(KIPO internal) & Keywords: copper dehydrogenase, mutation, multicopper oxidase, oxidase activity, dehydrogenase activity, L-DOPA		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y A	WANG, H. et al., "Crystal structures of multicopper oxidase CueO G304K mutant: structural basis of the increased laccase activity", Scientific Reports, 2018, vol. 8, article number 14252, pp. 1-12 abstract; pages 1-3, 8; figure 2	1-3 4-5,46
Y	HORAGUCHI, Y. et al., "Construction of Mutant Glucose Oxidases with Increased Dye-Mediated Dehydrogenase Activity", Int. J. Mol. Sci., 2012, vol. 13, pp. 14149-14157 abstract; pages 14151, 14156	1-3
A	US 2019-0382735 A1 (JIANGNAN UNIVERSITY) 19 December 2019 (2019-12-19) abstract; claims 1-5	1-5,46
A	MAZURENKO, I. et al., "Electrochemistry of copper efflux oxidase-like multicopper oxidases involved in copper homeostasis", Current Opinion in Electrochemistry, 2022, vol. 32, article number 100919, pp. 1-10 abstract	1-5,46
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "D" document cited by the applicant in the international application "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 19 September 2024		Date of mailing of the international search report 19 September 2024
Name and mailing address of the ISA/KR Korean Intellectual Property Office 189 Cheongsa-ro, Seo-gu, Daejeon 35208, Republic of Korea Facsimile No. +82-42-481-8578		Authorized officer HEO, Joo Hyung Telephone No. +82-42-481-5373

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SANDEEPA, S. et al., "Electrochemical detection of L-dopa using crude Polyphenol oxidase enzyme immobilized on electrochemically reduced RGO-Ag nanocomposite modified graphite electrode", Materials Science and Engineering B, 2018, vol. 232-235, pp. 15-21 whole document	1-5,46

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/031682

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13ter.1(a)),
☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/031682

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: **7,9,10,12,14,16-18,20,22,24,25,29,32,39,42,45,48,51,52,54,57,58,60-62,64-69,73-75**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Each of claims 7, 9, 10, 12, 14, 16-18, 20, 22, 24, 25, 29, 32, 39, 42, 45, 48, 51, 52, 54, 57, 58, 60-62, 64-69, 73-75 refers to a claim which is not drafted in accordance with Rule 6.4(a).
3. ☒ Claims Nos.: **6,8,11,13,15,19,21,23,26-28,30,31,33-38,40,41,43,44,47,49,50,53,55,56,59,63,70-72,76,77**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2024/031682

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
US	2019-0382735	A1	19 December 2019	CN	110106153	A	09 August 2019
				CN	110106153	B	29 December 2020
				US	11008552	B2	18 May 2021
