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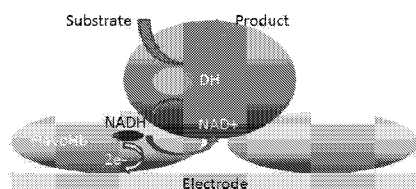
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(54) Title: ENZYMATIC ELECTRODE FOR REGENERATION OF OXIDISED AND/OR REDUCED FORMS OF PYRIDINE NUCLEOTIDES



(57) Abstract: The present invention relates to an electrode for regeneration of oxidised and/or reduced forms of pyridine nucleotides, said electrode characterized by having an electronically conducting surface to which a flavo-hemoglobin is adsorbed in the presence of an agent that directs enzyme orientation and wherein said flavohemoglobin is coupled to a pyridine nucleotide dependent enzyme. The invention further relates to a biosensor comprising the electrode of the present invention and to uses of said electrode and biosensor.

Figure 1



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Enzymatic electrode for regeneration of oxidised and/or reduced forms of pyridine nucleotides

Field of invention

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The present invention relates to an electrode for regeneration of oxidised and/or reduced forms of pyridine nucleotides, said electrode characterized by having an electronically conducting surface to which a flavohemoglobin is adsorbed in the presence of an agent that directs enzyme orientation and wherein said
10 flavohemoglobin is coupled to a pyridine nucleotide dependent enzyme. The invention further relates to a biosensor comprising the electrode of the present invention and to uses of said electrode and biosensor.

Background of invention

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Cellular metabolism and biological energy conversion rely on electron transfer (ET) reactions mediated by proteins and small non-protein molecules such as an NAD(P)H/NAD(P)⁺ redox couple sustaining electron and proton flows in enzymatic cascades (Nicholls, D. G.; Ferguson, S. J., Bioenergetics 3. Academic Press, Elsevier
20 Science: Amsterdam, 2002). Of those, biocatalytic recycling of NAD(P)H/ NAD(P)⁺, a soluble electron accepting cofactor of more than 400 NAD(P)⁺-dependent dehydrogenases (Gorton, L.; Dominguez, E., Reviews in Molecular Biotechnology 2002, 82 (4), 371-392), represents a true biotechnological challenge both in industrial biosynthesis (Schmid, A.; Dordick, J. S.; Hauer, B.; Kiener, A.; Wubbolts, M.; Witholt, B., Nature 2001, 409, 258-268) and electrochemical biosensor and biofuel cell
25 development (Gorton, L.; Domínguez, E., Electrochemistry of NAD(P)⁺/NAD(P)H. In Encyclopedia of Electrochemistry, Wiley-VCH Verlag GmbH & Co. KGaA: 2007). A substantial reaction overpotential, interference from other redox species and electrode fouling with reaction intermediates make direct electrochemical oxidation of NAD(P)H impractical (Moiroux, J.; Elving, P. J., J. Am. Chem. Soc. 1980, 102, 6533-6538), thus
30 triggering the search of advanced catalysts for NAD(P)H/NAD(P)⁺ recycling. While conductive polymer- and redox mediator-modified electrodes were extensively studied (Gorton, L.; Domínguez, E., Electrochemistry of NAD(P)⁺/NAD(P)H. In Encyclopedia of Electrochemistry, Wiley-VCH Verlag GmbH & Co. KGaA: 2007), just a few proteins
35 were shown to directly and at low overpotentials electrochemically convert NAD(P)H

into NAD(P)⁺. The examples are diaphorase (Kobayashi, D.; Ozawa, S.; Mihara, T.; Ikeda, T., *Denki Kagaku* 1992, 60 (12), 1056-1062); and the isolated I λ subcomplex of bovine mitochondrial NADH:ubiquinone oxidoreductase (Barker, C. D.; Reda, T.; Hirst, J., *Biochem.* 2007, 46, 3454-3464) and diaphorase fragment of NAD⁺-reducing [NiFe]-hydrogenase from *Ralstonia eutropha* (Lauterbach, L.; Idris, Z.; Vincent, K. A.; Lenz, O., *PLoS ONE* 2011, 6, e25939). Of those, the two latter systems, being isolated fragments of biological systems of a higher complexity, enable a reversible interconversion of NADH and NAD⁺. Recently, bacterial flavohemoglobin electronically wired to electrodes via Os complex-containing polymers as mediators of ET between the electrode and the pyrimidine nucleotides was shown to electrocatalytically oxidize NADH at potentials of the Os complexes used (Sosna, M.; Bonamore A.; Gorton L.; Boffi A.; Ferapontova, E. E., *Biosens. Bioelectron.* 2013, 42, 219-224). These ET mediators constitute a disadvantage since they represent an extra modification step, may be quite expensive in synthesis, dictate the potential of the redox reaction, which may be different from the inherent redox potential of the biocatalyst/pyridine nucleotide couple, can catalyze non-specific reactions of interfering compounds, and can undesirably affect enzyme activities, which results in quite high K_M values in reactions with their substrates.

Another problem with the present modified electrodes and electrochemical biosensors relates to high costs especially in relation to large scale production of biosensors. In addition, current electrodes for regeneration of oxidised and/or reduced forms of pyridine nucleotides are complex, display un-specificity for pyridine nucleotides (in particular, non-enzymatic modified electrodes) and comprise less stable enzyme complexes.

The present invention provides an electrode for regeneration of oxidised and/or reduced forms of pyridine nucleotides comprising a stable enzyme complex, displaying a high specificity for pyridine nucleotides and can function without mediators.

Summary of invention

It is within the scope of the present invention to provide an enzymatic electrode for regeneration of oxidised and/or reduced forms of pyridine nucleotides. The present

invention provides an electrode comprising a stable enzyme complex and displaying a high specificity for pyridine nucleotides.

5 Thus, one aspect of the present invention relates to an electrode for regeneration of oxidised and/or reduced forms of pyridine nucleotides, said electrode characterized by having an electronically conducting surface to which a flavohemoglobin or a derivative thereof is adsorbed in the presence of an agent that directs enzyme orientation and wherein said flavohemoglobin is coupled to a pyridine nucleotide dependent enzyme.

10 In one embodiment said flavohemoglobin is a bacterial flavohemoglobin. The flavohemoglobin is in one embodiment *E. coli* flavohemoglobin.

Said flavohemoglobin can be modified with the proviso that said flavohemoglobin remains catalytically active.

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It one embodiment of the present invention said pyridine nucleotide dependent enzyme is selected from NAD⁺- and (NADP)⁺-dependent enzyme.

20 In a preferred embodiment said pyridine nucleotide dependent enzyme is a dehydrogenase. Said dehydrogenase can for example be selected from the group consisting glucose dehydrogenase, lactate dehydrogenase, glutamate dehydrogenase, alcohol dehydrogenase, aldehyde dehydrogenase, galactose dehydrogenase and aspartate dehydrogenase

25 In another embodiment of the present invention said pyridine nucleotide dependent enzyme is modified with the proviso that said enzyme remains catalytically active.

It is preferred that said agent comprises a polypeptide conjugated to a fatty acid.

30 Preferably, said agent is a surfactant lipopeptide. Said surfactant lipopeptide is in one embodiment selected from the group consisting of polymyxin B, surfactin, daptomycin and iturin A

35 In one embodiment said oxidised and/or reduced forms of pyridine nucleotides are selected from the group consisting NAD⁺, NADH, NADP⁺, NADPH and analogues thereof.

It is preferred that said flavohemoglobin converts reduced forms of pyridine nucleotides into oxidised forms of pyridine nucleotides.

5 In one embodiment said electrically conducting surface comprises or consists of carbon or graphite. For example, said electrically conducting surface comprises or consists of a material selected from the group consisting of graphene oxide, reduced graphene, mesoporous carbon, carbon microfiber, carbon fiber, carbon paste, carbon cloth, carbon nanomaterial and carbon paper.

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In a preferred embodiment of the present invention said regeneration of oxidised and/or reduced forms of pyridine nucleotides takes place in the absence of an electron transfer mediator.

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Another aspect of the present invention relates to a method for production of food ingredients beverage ingredients and/or pharmaceutical additives said method comprising:

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- providing an electrode according to as described herein
- contacting the electrode with a solution comprising pyrimidine nucleotides and substrate that can be converted into product by the enzyme adsorbed on the electrode
- applying a potential to said electrode

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whereby pyridine nucleotide is oxidised by flavohemoglobin and substrate is transformed into a product by the enzyme using the oxidised pyridine nucleotide as a cofactor.

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Another aspect of the present invention relates to use of the electrode as described herein for production of food ingredients, beverage ingredients and/or pharmaceuticals.

Yet another aspect of the present invention relates to a biosensor comprising the electrode as defined above.

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A further aspect of the present invention relates to use of the biosensor as defined herien to measure the concentration of and/or detecting an analyte in a sample.

For example, said analyte is selected from the group consisting of blood ketones, hormones, steroids, amino-acids, lactate, sugars, alcohols, pharmaceuticals and pharmaceutical additives.

- 5 Another aspect of the present invention relates to a method for production of food ingredients, beverage ingredients, food analysis, medical biosensors, pharmaceutical synthesis and/or biosensing of food by applying the biosensor as defined herein.

10 A further aspect relates to a method of making an electrode according to the present invention comprising adsorption of flavohemoglobin /dehydrogenase on an electrode surface in the presence of a promoter that directs enzyme orientation.

Description of Drawings

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Figure 1. Schematic representation of flavohemoglobin (FlavoHb) and dehydrogenase (DH) assembly on the electrode surface in the presence of a promoter that directs flavohemoglobin orientation that is productive for the electrocatalytic oxidation of reduced forms of pyridine nucleotides produced during catalytic oxidation of the substrate of interest by dehydrogenase adsorbed on the flavohemoglobin layer

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Figure 2. Chemical structure of polymyxin B used as one of promoters of orientation.

Figure 3. Representative background-corrected cyclic voltammograms (CV) corresponding to electrocatalytic currents of NADH oxidation (1, 2 and 3 mM) at the Flavohemoglobin -modified electrodes, scan rate 70 mV s⁻¹. CVs were recorded in 20 mM Tris, pH 7.4, at a stationary electrode

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Figure 4. Dependences of the catalytic currents of NADH oxidation on the NADH concentration; derived from CVs, at (●) -200, (○) 70 mV. Solid lines are Sigma-Plot fitting of the data to the Michaelis-Menten equation

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Figure 5. Dependences of the catalytic currents of glucose oxidation on the glucose concentration at the Glucose dehydrogenase/Flavohemoglobin modified electrodes in the presence of 5 mM NAD⁺ and different concentrations of glucose in 20 mM Tris, pH 7.4, chronoamperometry at -200 and +70 mV. NAD⁺-dependent Glucose

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dyhydrogenase (*Bacillus* sp.) was immobilized by adsorption of enzyme onto the HMP electrode.

5 Detailed description of the invention

It is within the scope of the present invention to provide an enzymatic electrode for regeneration of oxidised and/or reduced forms of pyridine nucleotides. Such electrodes can for example be used in production of food ingredients, pharmaceutical synthesis and in biosensors. Current electrodes for regeneration of oxidised and/or reduced forms of pyridine nucleotides are complex, display un-specificity for pyridine nucleotides and comprise less stable enzyme complexes. The present invention provides an electrode comprising a stable enzyme complex and displaying a high specificity for pyridine nucleotides.

Thus, one aspect of the present invention relates to an electrode for regeneration of oxidised and/or reduced forms of pyridine nucleotides, said electrode characterized by having an electronically conducting surface to which a flavohemoglobin or a derivative thereof is adsorbed in the presence of an agent that directs enzyme orientation and wherein said flavohemoglobin is coupled to a pyridine nucleotide dependent enzyme.

The term "pyridine nucleotide dependent enzyme" as used herein refers to an enzyme that uses a pyridine nucleotide as a co-factor when converting its substrate into product. The term "enzyme" as used herein refers to the pyridine nucleotide dependent enzyme.

Flavohemoglobin

Flavohemoglobin belongs to the group of nitric oxide dioxygenase that are enzymes catalysing the conversion of nitric oxide (NO) to nitrate (NO₃⁻). The Enzyme Commission number (EC number) is 1.14.12.17.

Flavohemoglobins are widely represented among bacteria and yeast. The flavohemoglobins described herein can be from any organism comprising a gene encoding flavohemoglobin. For example, the flavohemoglobin as described herein is

from bacteria or yeast. In a preferred embodiment the flavohemoglobin is a bacterial flavohemoglobin.

Preferably, the flavohemoglobin is *E. coli* flavohemoglobin. *E. coli* flavohemoglobin has
5 SEQ ID NO:1. Thus, in a preferred embodiment the flavohemoglobin comprises an amino acid sequence having at least 70% sequence identity with SEQ ID NO:1, such as at least 75% sequence identity with SEQ ID NO:1, at least 80% sequence identity with SEQ ID NO:1, such as for example at least 85% sequence identity with SEQ ID NO:1, at least 90% sequence identity with SEQ ID NO:1, or such as for example at
10 least 95% sequence identity with SEQ ID NO:1.

Flavohemoglobin contains two domains: an N-terminal *b*-type heme-domain and a C-terminal NAD- and FAD-binding domain. Flavohemoglobins constitute a homogeneous group of proteins that share highly conserved active sites in both the heme- and
15 NAD/FAD-binding domains. The conserved amino acids within the heme domain include residues lining the heme pocket on both the proximal and distal sites, thus demonstrating a highly conserved region for ligand binding and/or for gaseous ligand diffusion. In addition, the amino acid residues responsible for flavin binding are also strictly conserved (Bonamore and Boffi; IUBMB Life, 60(1): 19–28, January 2008).

20 The conserved amino acids of the heme-domain correspond to the following amino acid residues of SEQ ID NO.1: Phe28, Tyr29, Phe43, Gln53, Leu57, Asn44, Val98, Leu102, and Tyr124 on the heme distal side and to His85, Glu135, Tyr95, and Lys84 on the heme proximal side. The three letters refer to the amino acid code and the
25 number refers to the amino acid number or position in the amino acid sequence. For example, Phe28 means phenylalanine at position 28 in the amino acid sequence.

Thus, in a preferred embodiment, the flavohemoglobin comprises an amino acid sequence wherein at least 10 amino acids, such as at least 11 amino acids or at least
30 12 amino acids residues corresponding to Phe28, Tyr29, Phe43, Gln53, Leu57, Asn44, Lys84, His85, Tyr95, Val98, Leu102, Tyr124 and Glu135 of SEQ ID NO.1 are conserved.

Preferably, the flavohemoglobin comprises an amino acid sequence wherein amino acids residues corresponding to Phe28, Tyr29, Phe43, Gln53, Leu57, Asn44, Lys84, His85, Tyr95, Val98, Leu102, Tyr124 and Glu135 of SEQ ID NO.1 are conserved.

- 5 Conserved amino acid residues at the FAD/NAD binding domain corresponds to the following amino acid residues of SEQ ID NO.1: Phe290, Gly270, Glu388, Thr272, Pro273, Gly186, Gln187, Tyr188, and Ser232.

- 10 In a preferred embodiment, the flavohemoglobin comprises an amino acid sequence wherein at least 6, such as at least 7 or at least 8 of the amino acids residues corresponding to Phe290, Gly270, Glu388, Thr272, Pro273, Gly186, Gln187, Tyr188, and Ser232 of SEQ ID NO.1 are conserved.

- 15 Preferably, the flavohemoglobin comprises an amino acid sequence, wherein amino acids residues corresponding to Phe290, Gly270, Glu388, Thr272, Pro273, Gly186, Gln187, Tyr188, and Ser232 of SEQ ID NO.1 are conserved.

- 20 In a preferred embodiment the flavohemoglobin comprises an amino acid sequence wherein at least 16 amino acids, such as at least 17 amino acids, at least 18 amino acids, such as at least 19 amino acids, at least 20 amino acids or at least 21 amino acids residues corresponding to Phe28, Tyr29, Phe43, Gln53, Leu57, Asn44, Lys84, His85, Tyr95, Val98, Leu102, Tyr124, Glu135, Phe290, Gly270, Glu388, Thr272, Pro273, Gly186, Gln187, Tyr188, and Ser232 of SEQ ID NO.1 are conserved.

- 25 In a particular embodiment the flavohemoglobin comprises an amino acid sequence wherein amino acids residues corresponding to Phe28, Tyr29, Phe43, Gln53, Leu57, Asn44, Lys84, His85, Tyr95, Val98, Leu102, Tyr124, Glu135, Phe290, Gly270, Glu388, Thr272, Pro273, Gly186, Gln187, Tyr188, and Ser232 of SEQ ID NO.1 are conserved.

- 30 In a preferred embodiment thereof, the flavohemoglobin comprises an amino acid sequence having at least 90% sequence identity with SEQ ID NO:1 provided that amino acids residues corresponding to Phe28, Tyr29, Phe43, Gln53, Leu57, Asn44, Lys84, His85, Tyr95, Val98, Leu102, Tyr124, Glu135, Phe290, Gly270, Glu388, Thr272, Pro273, Gly186, Gln187, Tyr188, and Ser232 of SEQ ID NO.1 are conserved.

In a another preferred embodiment thereof, the flavohemoglobin comprises an amino acid sequence having at least 95% sequence identity with SEQ ID NO:1 provided that amino acids residues corresponding to Phe28, Tyr29, Phe43, Gln53, Leu57, Asn44, Lys84, His85, Tyr95, Val98, Leu102, Tyr124, Glu135, Phe290, Gly270, Glu388, Thr272, Pro273, Gly186, Gln187, Tyr188, and Ser232 of SEQ ID NO.1 are conserved.

The electrode comprises a flavohemoglobin or a derivative thereof. By derivative thereof is for example meant modified flavohemoglobin. The flavohemoglobin is in one embodiment modified with the proviso that said flavohemoglobin remains catalytically active. For example, the flavohemoglobin may be chemically or physically modified with the proviso that said flavohemoglobin remains catalytically active. Preferably, the flavohemoglobin is genetically modified. The flavohemoglobin may for example be tagged. Thus, in one embodiment the flavohemoglobin is a recombinant protein.

For example the flavohemoglobin can be modified by substituting, deleting, inserting and/or modifying one, two, three, four or further amino acids. Preferably, the flavohemoglobin can be modified by conservative amino acid substitutions.

Substitutions within the groups of amino acids, shown below, are considered conservative amino acid substitutions. Substitutions between the different groups of amino acids are considered non-conservative amino acid substitutions.

P, A, G, S, T (neutral, weakly hydrophobic)

Q, N, E, D, B, Z (hydrophilic, acid amine)

H, K, R (hydrophilic, basic)

F, Y, W (hydrophobic, aromatic)

L, I, V, M (hydrophobic)

C (cross-link forming)

The present invention discloses that flavohemoglobin directly and at low overpotentials electrochemically converts NAD(P)H into NAD(P)⁺. The term "overpotential" as used herein refers to the potential.

In a preferred embodiment, said flavohemoglobin converts reduced forms of pyridine nucleotides into oxidised forms of pyridine nucleotides. Preferably, said oxidised and/or

reduced forms of pyridine nucleotides are selected from the group consisting NAD^+ , NADH , NADP^+ , NADPH and analogues thereof.

5 Flavohemoglobin is capable of detoxifying nitric oxide (NO). In reaction with oxygen and NADH , flavohemoglobin demonstrates a genuine NO dioxygenase activity, by reducing oxygen to superoxide at the heme site with a concomitant oxidation of NADH at the FAD site (Figure 1B). Electrons from NADH are relayed by FAD to heme and further to the iron-bound oxygen via intramolecular electron transfer (ET).

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Pyridine nucleotide dependent enzyme

15 The electrode of the present invention comprises a flavohemoglobin coupled to a pyridine nucleotide dependent enzyme. Thereby, the flavohemoglobin enzyme can generate NAD^+ or NADP^+ that is reduced by the pyridine nucleotide dependent enzyme to NADH or NADPH while converting its substrate into a product (Figure 1)

20 It is preferred that said regeneration of oxidised and/or reduced forms of pyridine nucleotides takes place in the absence of an electron transfer mediator.

The pyridine nucleotide is preferably selected from the group consisting of NAD^+ , NADH , NADP^+ , NADPH . In a preferred embodiment the enzyme is selected from $\text{NAD}^{+/-}$ and $(\text{NADP})^{+/-}$ -dependent enzymes.

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The pyridine nucleotide dependent enzyme may for example be a modified enzyme. The enzyme is in one embodiment modified with the proviso that said enzymes remains catalytically active. For example, the enzymes may be chemically or physically modified with the proviso that said enzymes remains catalytically active. Preferably, the enzyme is genetically modified. The enzymes may for example be tagged. Thus, in one embodiment the enzyme is a recombinant protein.

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For example the pyridine nucleotide dependent enzyme can be modified by substituting, deleting, inserting and/or modifying one, two, three, four or further amino acids. Preferably, the pyridine nucleotide dependent enzyme can be modified by

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conservative amino acid substitutions. Conservative amino acid substitutions are defined herein above.

5 The pyridine nucleotide dependent enzyme may for example be selected from the group of enzymes having Enzyme Commission number (EC) 1.1.1.X; 1.2.1.X; 1.3.1.X; 1.4.1.X; 1.5.1.X; 1.7.1.X; 1.8.1.X; 1.10.1.X; 1.11.1.1; 1.11.1.2; 1.12.1.X; 1.14.12.X ; 1.14.13.X; 1.14.21; 1.14.99.9; 1.14.99.10; 1.16.1.X; 1.17.1.X; 1.18.1.X; 1.20.1.X; 1.22.1.X. The X denotes an arbitrary digit. For example 1.1.1.X includes all groups starting with 1.1.1.

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In another embodiment the pyridine nucleotide dependent enzyme may for example be selected from the group of enzymes having Enzyme Commission numbers (EC) 1.1.1; 1.2.1; 1.3.1; 1.4.1; 1.5.1; 1.7.1; 1.8.1; 1.10.1; 1.11.1.1; 1.11.1.2; 1.12.1; 1.16.1; 1.17.1; 1.18.1; 1.20.1;; 1.22.1.

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The pyridine nucleotide dependent enzyme may for example be selected from the group of enzymes having Enzyme Commission number (EC) 1.1.1; 1.2.1; 1.3.1; 1.4.1; 1.5.1; 1.7.1; 1.8.1; 1.10.1; 1.11.1.1; 1.11.1.2; 1.12.1;; 1.14.12 ; 1.14.13; 1.14.21; 1.16.1; 1.17.1; 1.18.1; 1.20.1; and/or 1.22.1.

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In a preferred embodiment the pyridine nucleotide dependent enzyme is a pyridine nucleotide dependent dehydrogenase. In a preferred embodiment said dehydrogenase is selected from the group consisting glucose dehydrogenase, lactate dehydrogenase, glutamate dehydrogenase, alcohol dehydrogenase, aldehyde dehydrogenase, galactose dehydrogenase and aspartate dehydrogenase

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Agent that directs enzyme orientation

30 The flavohemoglobin is attached to an electronically conducting surface in the presence of an agent that directs enzyme orientation. Thus, the agent directs enzyme orientation of the flavohemoglobin enzyme at the electrode. When flavohemoglobin is properly oriented at the electrode it is capable of reversibly electrocatalyze interconversion of NADH and NAD⁺ or NADPH and NADP⁺ thus paving the way to direct monitoring of its ET reactions and development of technologically relevant bio-electrocatalysts for cofactor regeneration. The enzyme is for example oriented at the

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electrode via its heme domain thus enabling a reversible catalytic transformation of NADH/NAD⁺ at the FAD site upon applying a proper potential (see example section and Figure 1). The agent can also anchor the flavohemoglobin enzyme to the electrode surface as described below.

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In a preferred embodiment said agent comprises a polypeptide conjugated to a fatty acid. Thereby, the agent can interact with a hydrophilic electrode surface and with the heme group of flavohemoglobin, thereby functioning as an anchor between the electrode surface and the flavohemoglobin. Preferably, said agent is a surfactant lipopeptide. Said surfactant lipopeptide is for example selected from the group consisting of polymyxin B, surfactin, daptomycin and iturin A. In a particular embodiment the agent is polymyxin B (PMB). PMB is a small polypeptide conjugated to a fatty acid and behaves as a cationic detergent at physiological pH and can function as an anchor as described in the example section.

15

Electrochemical cell

The present invention also provides an electrochemical cell comprising the electrode as defined herein.

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Electrochemical cells for use in accordance with the invention may be constructed according to conventional methods known in the art (A. J. Bard and L.F. Faulkner, *Electrochemical Methods. Fundamentals and Applications*. John Wiley & Sons, 2001)

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Typically, the electrochemical cell may be constructed comprising a working electrode, a counter electrode (also referred to as an auxiliary electrode) and a reference electrode immersed in the same electrolyte solution or in different electrolyte solutions. Thus, the working and counter electrodes are in electrical contact with the reference electrode either by immersing the electrodes in the same electrolyte solution or by immersing the working and counter electrodes in an electrolyte solution which is electrically linked to the reference electrode electrolyte by a salt-bridge or a semi-permeable membrane.

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The working electrode, as referred to herein, is the electrode to which flavohemoglobin coupled to the pyrimidine nucleotide dependent enzyme is adsorbed. Thus, the working

electrode is the electrode according to the present invention. The working electrode or the electrode as defined herein comprises an electrically conducting surface to which flavohemoglobin is attached or adsorbed. It is preferred that the flavohemoglobin is attached or anchored via a polypeptide conjugated to a fatty acid as described above.

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The reference electrode can be any commonly used reference electrode such as for example a hydrogen electrode, a calomel electrode or copper/copper(II) sulfate electrode. In a preferred embodiment the reference electrode is a silver chloride (Ag/AgCl) electrode. The counter electrode can be any commonly used counter electrodes such as for example platinum, stainless steel, carbon electrodes. In a preferred embodiment the counter electrode is either a Pt electrode or a stainless steel electrode.

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The electrochemical cell may also be constructed comprising only a working and an counter electrode immersed in the same electrolyte solution or in different electrolyte solutions separated by a semipermeable membrane. The auxiliary electrode functions as a cathode whenever the working electrode is operating as an anode and vice versa.. The potential of the auxiliary electrode is usually not measured and is adjusted so as to balance the reaction occurring at the working electrode. Auxiliary electrodes are often fabricated from electrochemically inert materials such as gold, platinum, or carbon.

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The electrically conducting surface of the electrode according to the present invention may for example comprise or consist of carbon or graphite. In one embodiment said electrically conducting surface comprises or consists of a material selected from the group consisting of spectroscopic graphite, highly oriented pyrolytic graphite, graphene oxide, reduced graphene, mesoporous carbon, carbon microfiber, carbon fiber, carbon paste, carbon cloth, screen-printed carbon, carbon nanomaterials and carbon paper.

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The electrolyte solution comprises charge carriers are ions, atoms or molecules that have gained or lost electrons so they are electrically charged. The charge carriers may be selected from the group consisting of protons, hydroxide ions, metal ions, halide ions, ammonium ions and oxyanions. Oxyanions may include nitrate ions, sulphate ions and phosphate ions. In a preferred embodiment the electrolyte solution is a salt water solution. For example, the electrolyte may comprise NaCl.

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The electrolyte solution may further comprise a pH buffer such as for example HEPES or Tris. Preferably, the electrolyte solution has a pH at which the enzymes adsorbed at the electrode of the invention are stable. Preferably the electrolyte solution is maintained at a pH of between 5 and 10 and more preferably between 7 and 8.

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In the electrolytic cell as described herein the current flowing with the working electrode at a given potential relative to the reference electrode, may be used as a quantitative measure of the amount of oxidized or reduced pyridine nucleotide present in the electrolyte. The current flowing in the electrochemical cell can be related to the concentration of the oxidized or reduced form of the pyridine nucleotide present in the electrolyte through equations which are well known in the art.

10

When applying a proper potential to the electrochemical cell a reversible catalytic transformation of NAD(P)H/NAD(P)⁺ takes place at the FAD site of flavohemoglobin. This electrocatalytic oxidation of NAD(P)H by flavohemoglobin starts from potentials of the Fe²⁺/Fe³⁺ redox couple of the heme domain. Electrocatalytic oxidation of NAD(P)H by flavohemoglobin and the reduction of NAD(P)⁺ by the pyrimidine dependent enzyme may preferably start from around -200 mV versus a reference electrode such as for example the Ag/AgCl reference electrode. Accordingly, biotransformation and analysis of enzyme substrates may start at very low potentials such as 0 mV.

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The electrode of the present invention displays a fast shuttling of electrons between the electrode surface, the heme domain and the FAD domain of flavohemoglobin. The internal electron transfer from the heme domain to the FAD domain is dependent on NAD(P)⁺. The internal ET rate between the heme domain and the FAD domain (k_{intramol}) is for example about 150 s⁻¹, whereas the ET rate between electrode and heme-domain (k_s) is about 20 s⁻¹ (see example section).

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The working electrode may be rotated to ensure that a continuous supply of reactants to the electrode/electrolyte interface is maintained. The speed of rotation of the working electrode may for example be between 0 and 5000 revolutions per minute (rpm), such as between 200 and 3000 rpm, for example between 500 and 2000 rpm. In a preferred embodiment the speed of rotation of the working electrode is about 1000 rpm.

30

The supply of the reactants to the electrode/electrolyte interface can be also maintained by a flow-injection system with a peristaltic pump (Lindgren, A., Munteanu, F., Gazaryan, I., Ruzagas, T., Gorton, L, J. Electroanal. Chem. 458 (1998) 113-120) or within the (micro)fluidic system device

5

The electrochemical cell can for example function as a voltammetric transducer where cell current is recorded as a function of the applied potential thereby generating a voltammogram. The current flowing in the electrochemical cell can be related to the concentration of the oxidized or reduced form of the pyridine nucleotide present in the electrolyte through equations which are well known in the art. The electrochemical cell may for example employ cyclic voltammetry, differential pulse voltammetry, square wave voltammetry, impedance spectroscopy, chronoamperometry, or chronocoulometry. In another embodiment the electrochemical cell employs cyclic voltammetry and amperometry.

10

As described above, the flavohemoglobin-enzyme complex adsorbed on the electrode surface exhibits improved stability when compared to known electroenzymatic systems. In addition, the electrode or the electrochemical cell according to the present invention can be produced at low costs and in large scale making them particularly suitable for industries requiring large scale manufacturing such as the food and medical industry.

15

Thus, another aspect of the present invention relates to use of the electrode as described herein for production of food ingredients, beverage ingredients and/or pharmaceuticals. In particular, the electrochemical cell may be used for production of food ingredients and/or beverage ingredients.

20

In this use of the electrode, a potential is applied to the electrode to which the flavohemoglobin-enzyme complex is adsorbed, such that pyridine nucleotide is converted from its reduced to its oxidised form by the flavohemoglobin-enzyme complex. The enzyme may use the oxidised form of the pyridine nucleotide as a cofactor when transforming its substrate into a product. It is this regeneration of the oxidized form of the pyridine nucleotide by flavohemoglobin that enables the enzyme to maintain turnover of substrate to products.

25

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30

By specific selection of the enzyme coupled to flavohemoglobin, a wide range of reactions mediated by pyrimidine dependent enzymes can be controllably driven in solution to form useful or desirable products.

- 5 Further embodiments include use of the electrode for biotransformation of alpha-keto amino acids to L-aminoacid using a NAD⁺-dependent amino acid dehydrogenase; use of the electrode for reduction of alpha-keto amino acids or oxidation of alcohols into hydroxy acids using a dehydrogenase; use of the electrode for reduction of ketones or aldehydes to alcohols using alcohol dehydrogenase; use of the electrode for
- 10 conversion of sugars into alcohols using dehydrogenase; use of the electrode for biotransformation of diols into chiral lactones using alcohol dehydrogenases.

The present invention also provides a method for production of food ingredients beverage ingredients and/or pharmaceutical additives by applying the electrode as

15 described herein. The method may comprise the following steps:

- providing an electrode or an electrochemical cell as described herein
- contacting the electrode with a solution comprising pyrimidine nucleotides and substrate that can be converted into product by the enzyme adsorbed on the electrode
- 20 – applying a potential to said electrode

whereby pyridine nucleotide is oxidised by flavohemoglobin and substrate is transformed into a product by the enzyme using the oxidised pyridine nucleotide as a cofactor.

25 It is preferred that the substrate can be used as food ingredients, beverage ingredients and/or as pharmaceutical additives. Examples of substrates and products are described above.

30 *Biosensor*

The electrode or the electrochemical cell according to the present invention may also be used to measure the concentration of fluid analytes such as for example analytes present in food. The measurement can be performed by voltammetric or amperometric

35 measurements as described herein above.

Another aspect of the present invention relates to a biosensor comprising the electrode as defined above. In a preferred embodiment the electrode comprises flavohemoglobin coupled to a pyridine nucleotide dependent enzyme. Preferably said enzyme is a
5 dehydrogenase. In another preferred embodiment the flavohemoglobin of the biosensor is a bacterial flavohemoglobin such as *E. coli* flavohemoglobin.

It is appreciated that the biosensor comprises an electrochemical cell as described above. Thus, the biosensor comprises a working electrode and a reference electrode
10 as described above. The biosensor may in one embodiment further comprise an auxiliary electrode.

A further aspect of the present invention relates to use of the biosensor in food and/or medical analysis.

15 In this aspect, the biosensor is used to measure the concentration of and/or detecting an analyte in a sample. Thus, a further aspect of the invention relates to a method for measuring the concentration of and/or detecting an analyte in a sample by applying the biosensor according to the present invention. As described above, biosensor
20 applications may also include the direct detection of pyridine nucleotides and measurement of their concentration in a solution.

In one embodiment the food analyte is selected from the group consisting of blood ketones, hormones, steroids, amino-acids, lactate, sugars, alcohols, pharmaceuticals
25 and pharmaceutical additives. When measuring the concentration of and/or detecting said analyte an enzyme may be chosen which converts the analyte into a product with concomitant transformation of a pyridine nucleotide from its oxidised form to its reduced form (or alternatively from its reduced form to its oxidised form).

The original form of the pyridine nucleotide is regenerated by the enzyme adsorbed
30 with the flavohemoglobin on the electrode surface. The current flowing in the electrolytic cell can be related to the concentration of one form of the pyridine nucleotide in the electrolytic solution and hence to the concentration of the enzyme substrate in the electrolytic solution.

Method of making the electrode

Another aspect of the present invention relates to a method of making an electrode as described herein comprising adsorption of flavohemoglobin and enzyme on an
5 electrode surface in the presence of an agent that directs flavohemoglobin orientation.

The flavohemoglobin, the enzyme and the agent that directs flavohemoglobin orientation is as defined herein above. In a preferred embodiment the enzyme is a dehydrogenase.

10 In a preferred embodiment the method comprises stepwise adsorption of flavohemoglobin and enzyme on the electrode surface.

The method may further comprise crosslinking of the flavohemoglobin-enzyme
15 complex with a bifunctional crosslinking reagent. An example of a bifunctional crosslinking reagent is glutaraldehyde.

In a further embodiment, the method further comprises applying a covering polymer membrane assembly over the flavohemoglobin-enzyme layer. The flavohemoglobin-
20 enzyme layer is a layer of flavohemoglobin-enzyme complexes adsorbed on the electrode surface. An example of a covering polymer membrane is Nafion.

The method may also comprise the application of a polymer membrane with chemically attached pyridine nucleotides. Thus, in another embodiment the method comprises
25 stepwise adsorption of flavohemoglobin, a polymer membrane with chemically attached pyridine nucleotides, and enzyme on the electrode surface. Examples of a polymer membrane with chemically attached pyridine nucleotides are polyethylene imine, polylysine, and polyethyleneglycol.

30 This embodiment of the method may be combined with crosslinking of the flavohemoglobin-enzyme-polymer layer with a bifunctional crosslinking reagent. The flavohemoglobin-enzyme-polymer layer is a layer of polymer membrane with chemically attached pyridine nucleotides and flavohemoglobin-enzyme complexes adsorbed on the electrode surface. The method can be further combined with covering
35

Examples

Electrocatalytic oxidation/reduction of pyrimidine nucleotides by flavohemoglobin coupled with a dehydrogenase

Adsorption of flavohemoglobin on graphite results in the bioelectrocatalytically non-productive orientation. NAD⁺/NADH electrocatalytically active *E. coli* flavohemoglobin films were obtained by co-adsorption with polymyxin B (PMB) (Figure 2) acting as a cationic detergent at physiological pH. As such, it productively interacts both with the hydrophilic graphite surface and with the hydrophobic heme domain of flavohemoglobin known for its ability to specifically bind lipids. Graphite electrodes were modified by applying a 5 μ L of a 10:1 v/v mixture of a 4.1 mg ml⁻¹ solution of HMP and a 2.6 mg ml⁻¹ solution of PMB, both in aqueous Tris-HCl, pH 7.5, on the electrodes surface for a 4 h adsorption at 4 °C under the lid. A schematic representation of flavohemoglobin (FlavoHb) and dehydrogenase (DH) assembly on the electrode surface in the presence of an agent that directs enzyme orientation such as PMB is shown in Figure 1. Voltammetric responses from flavohemoglobin co-adsorbed with PMB, at -202 mV, could be correlated with oxidation/reduction of the heme active site, and were characterized by the ET rate constant, k_s , of about 20 s⁻¹. The protein surface coverage of 8.6 pmol cm⁻² was close to the theoretical monolayer.

Electroenzymatic oxidation of NADH by flavohemoglobin started from the redox potentials of the heme active site (Figure 3), simultaneously, the electroenzymatic reduction of NAD⁺ starting from the same potentials occurred.

Rotating disk electrode experiments with flavohemoglobin/PMB complex adsorbed on graphite demonstrated that limiting catalytic currents of NADH oxidation were independent of the rotation rate, which implied a kinetic control of the electroenzymatic reaction. The bioelectrocatalytic currents were proportional to the NADH concentration and followed the Michaelis-Menten dependence with a K_M of 1.68 mM at 70 mV and 2.28 mM at -200 mV (Figure 4). Those values were higher than 3.2 μ M reported for NADH oxidation in solution and lower than those shown with Os redox polymers as ET mediators. The catalytic rate constant of 2.4 s⁻¹ was lower than 94 s⁻¹ reported for the solution catalysis with O₂ as electron acceptor and reflected the effects of enzyme

immobilization and replacement of the natural electron acceptor by the electrode. The bioelectrocatalytic currents of NAD^+ reduction were proportional to the concentrations of NAD^+ .

- 5 When NAD^+ -dependent glucose dehydrogenase was physically adsorbed onto the Flavohemoglobin/PMB-modified graphite electrodes, by adsorption of 5 μL of a 5 mg mL^{-1} aqueous solution of glucose dehydrogenase (*Bacillus* sp.) placed onto the Flavohemoglobin/PMB modified surface for 1 h at 4 $^{\circ}\text{C}$, bioelectrocatalytic signals corresponding to the electrocatalytic oxidation of NAD^+ reduced to NADH during enzymatic oxidation of glucose by glucose dehydrogenase, could be
- 10 chronoamperometrically followed at -200 and +70 mV in the presence of NAD^+ and upon additions of different concentrations of glucose (Figure 5). The bioelectrocatalytic currents were proportional to the concentration of added glucose, which allowed its quantitative determination within 1-5 mM concentration range important for clinical
- 15 analysis of glucose levels in diabetic patients, without interference from some other redox species that may be present in blood samples (uric and ascorbic acids).

Sequences

20 **SEQ ID NO: 1**

Amino acid sequence

UniProtKB - P24232 (HMP_ECOLI)

MLDAQTIATVKATIPLLVETGPKLTAHFYDRMFTHNPELKEIFNMSNQRNGDQREALF
25 NAIAAYASNIENLPALLPAVEKIAQKHTSFQIKPEQYNIVGEHLLATLDEMFSFGQEVLD
AWGKAYGVLANFINREAEIYNENASKAGGWEGTRDFRIVAKTPRSALITSFELEPVDG
GAAEYRPGQYLGVLKPEGFPHQEIRQYSLTRKPDGKGYRIVKREEGGQVSNWLHN
HANVGDVVKLVAPAGDFFMAVADDTPTLISAGQTPMLAMLDLAKAGHTAQVNWFFH
AAENGDVHAFADDEVKLGQSLPRFHTWYRQPSERAKGQFDSEGLMDLSKLEGAFSD
30 PTMQFYLCGPVGFQM TAKQLVDLGV KQENIHYESCFGPHKVL

Claims

1. An electrode for regeneration of oxidised and/or reduced forms of pyridine nucleotides, said electrode characterized by having an electronically conducting surface to which a flavohemoglobin or a derivative thereof is adsorbed in the presence of an agent that directs enzyme orientation and wherein said flavohemoglobin is coupled to a pyridine nucleotide dependent enzyme.
2. The electrode according to claim 1, wherein said flavohemoglobin is a bacterial flavohemoglobin.
3. The electrode according to any of claims 1 and 2, wherein said flavohemoglobin is *E. coli* flavohemoglobin.
4. The electrode according to any one of the preceding claims, wherein said flavohemoglobin is modified with the proviso that said flavohemoglobin remains catalytically active.
5. The electrode according to any one of the preceding claims, wherein said pyridine nucleotide dependent enzyme is selected from NAD⁺- and (NADP)⁺- dependent enzyme.
6. The electrode according to any one of the preceding claims, wherein said pyridine nucleotide dependent is a dehydrogenase.
7. The electrode according to any one of the preceding claims, wherein said dehydrogenase is selected from the group consisting glucose dehydrogenase, lactate dehydrogenase, glutamate dehydrogenase, alcohol dehydrogenase, aldehyde dehydrogenase, galactose dehydrogenase and aspartate dehydrogenase.
8. The electrode according to any one of the preceding claims, wherein said pyridine nucleotide dependent enzyme is modified with the proviso that said enzyme remains catalytically active.

9. The electrode according to any one of the preceding claims, wherein said agent comprises a polypeptide conjugated to a fatty acid.
10. The electrode according to any one of the preceding claims, wherein said agent is a surfactant lipopeptide.
11. The electrode according to claim 6, wherein said surfactant lipopeptide is selected from the group consisting of polymyxin B, surfactin, daptomycin and iturin A
12. The electrode according to any one of the preceding claims, wherein said oxidised and/or reduced forms of pyridine nucleotides are selected from the group consisting of NAD^+ , NADH , NADP^+ , NADPH and analogues thereof.
13. The electrode according to any one of the preceding claims, wherein said flavohemoglobin converts reduced forms of pyridine nucleotides into oxidised forms of pyridine nucleotides.
14. The electrode according to any one of the preceding claims, wherein said electrically conducting surface comprises or consists of carbon or graphite.
15. The electrode according to any one of the preceding claims, wherein said electrically conducting surface comprises or consists of a material selected from the group consisting of graphene oxide, reduced graphene, mesoporous carbon, carbon microfiber, carbon fiber, carbon paste, carbon cloth, carbon nanomaterial and carbon paper.
16. The electrode according to any one of the preceding claims, wherein said regeneration of oxidised and/or reduced forms of pyridine nucleotides takes place in the absence of an electron transfer mediator.
17. A method for production of food ingredients beverage ingredients and/or pharmaceutical additives said method comprising:
- providing an electrode according to any one of claims 1 to 16

- contacting the electrode with a solution comprising pyrimidine nucleotides and substrate that can be converted into product by the enzyme adsorbed on the electrode
- applying a potential to said electrode

5 whereby pyridine nucleotide is oxidised by flavohemoglobin and substrate is transformed into a product by the enzyme using the oxidised pyridine nucleotide as a cofactor.

10 18. Use of the electrode according to any one of claims 1 to 16 for production of food ingredients, beverage ingredients and/or pharmaceuticals.

19. A biosensor comprising the electrode according to any one of claims 1 to 16.

15 20. Use of the biosensor according to claim 12 to measure the concentration of and/or detecting an analyte in a sample.

21. The use according to claims 20, wherein said analyte is selected from the group consisting of blood ketones, hormones, steroids, amino-acids, lactate, sugars, alcohols, pharmaceuticals and pharmaceutical additives.

20 22. A method for production of food ingredients, beverage ingredients, food analysis, medical biosensors, pharmaceutical synthesis and/or biosensing of food by applying the biosensor according to claim 19.

25 23. A method of making an electrode according to any of claims 1 to 16 comprising adsorption of flavohemoglobin /dehydrogenase on an electrode surface in the presence of a promoter that directs enzyme orientation.

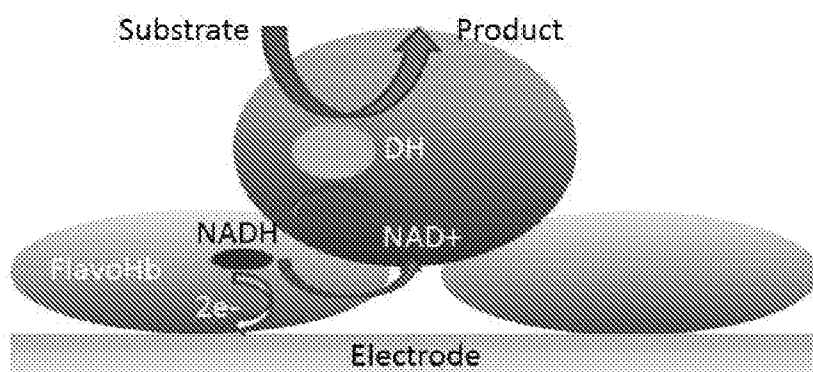


Figure 1

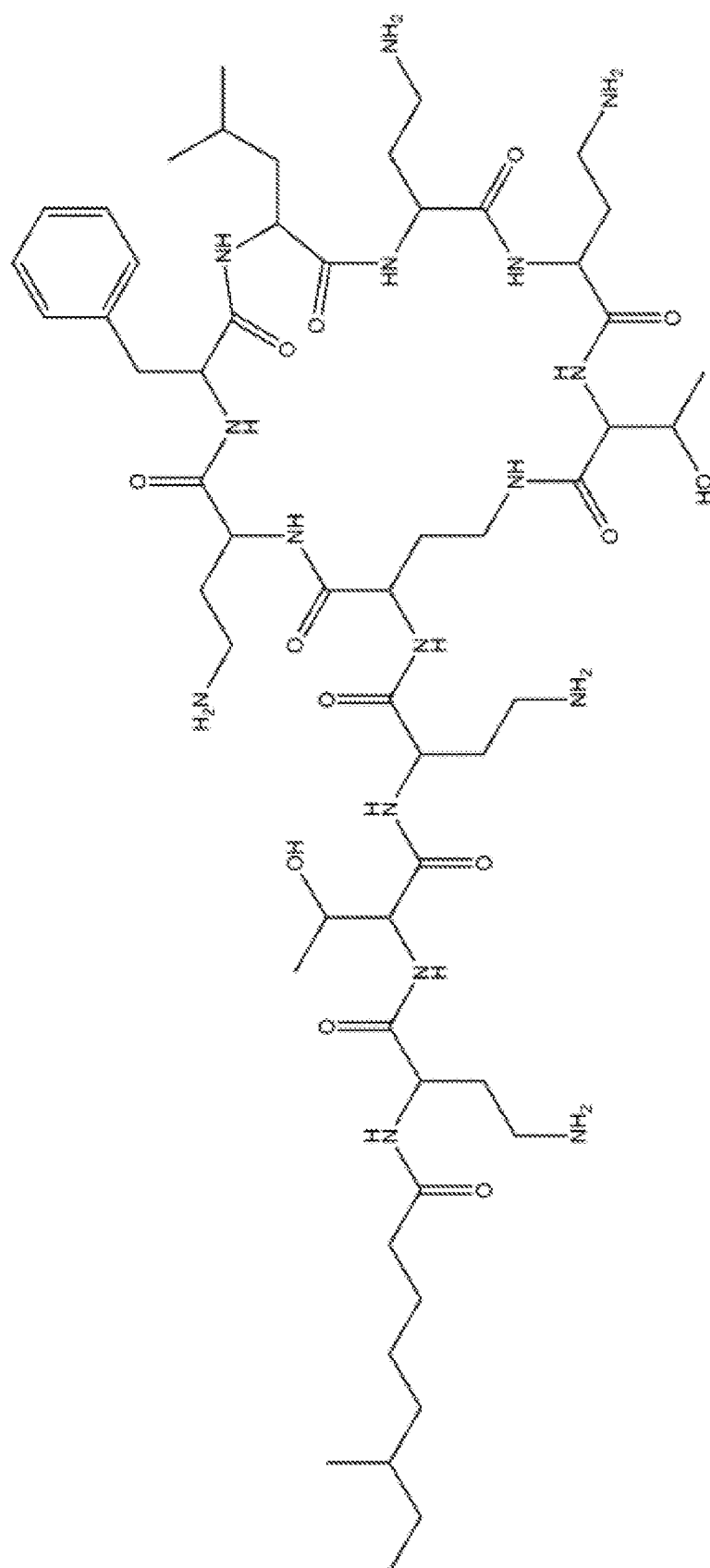


Figure 2

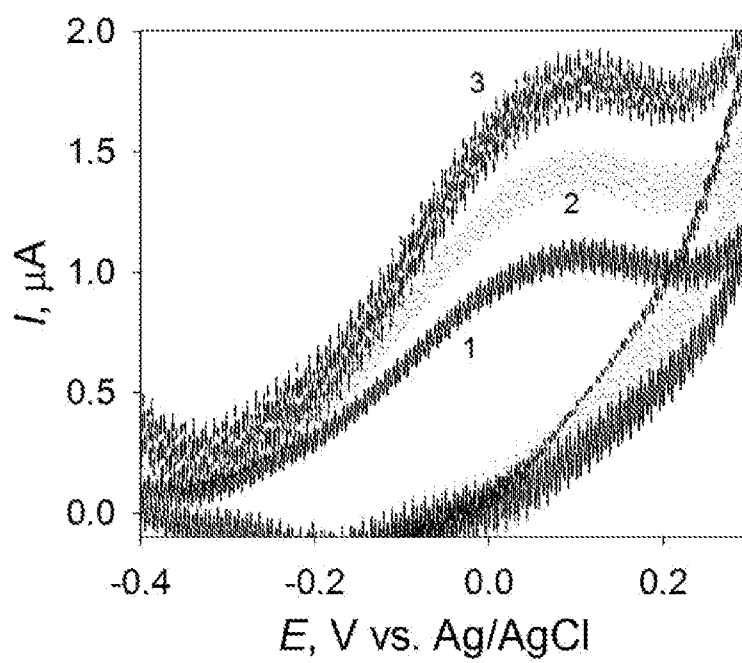


Figure 3

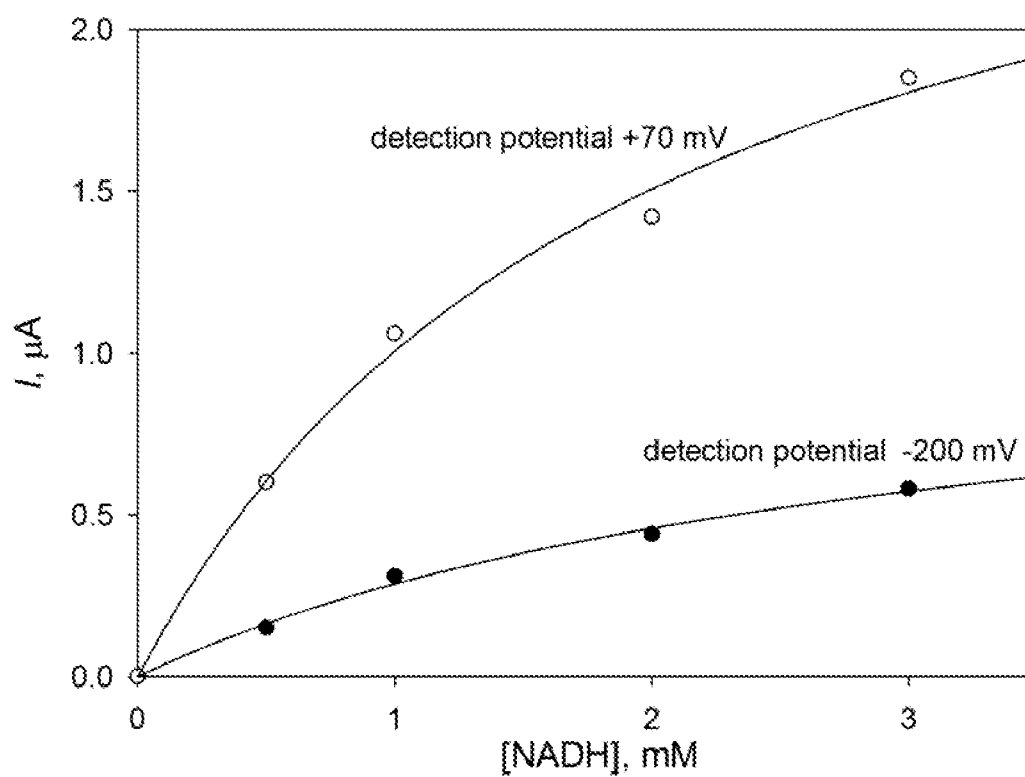


Figure 4

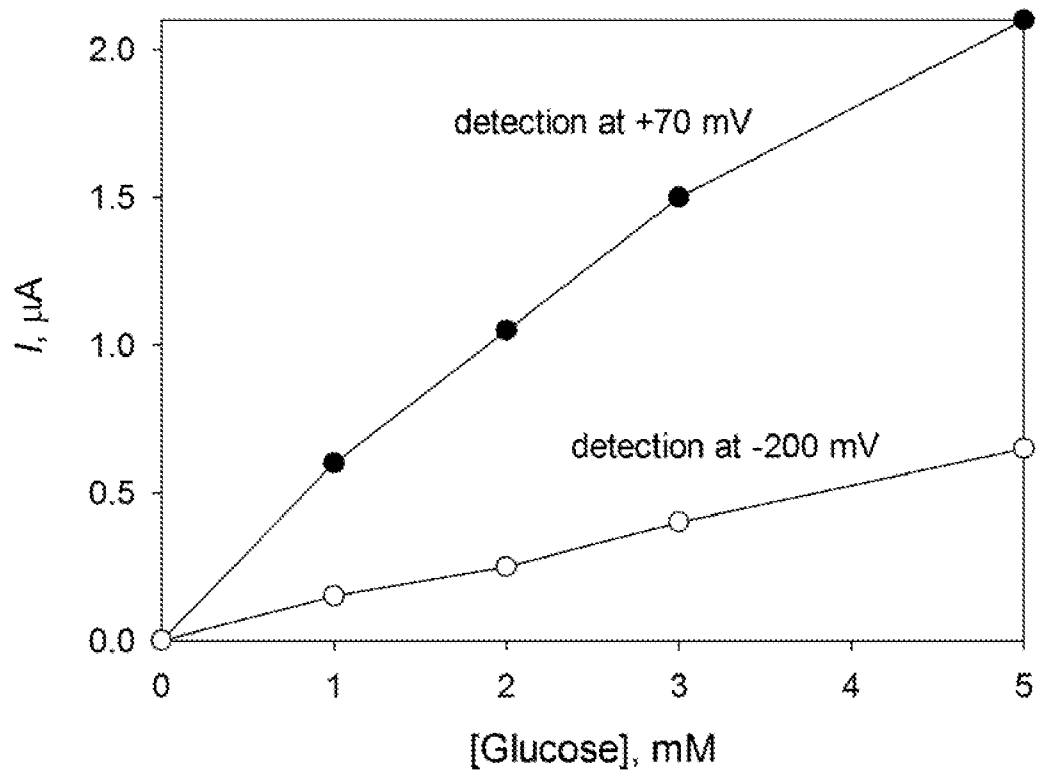


Figure 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2016/050246

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/26
ADD.

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)
C12Q

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

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

EPO-Internal, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MACIEJ SOSNA ET AL: "Direct electrochemistry and Os-polymer-mediated bioelectrocatalysis of NADH oxidation by Escherichia coli flavohemoglobin at graphiteelectrodes", BIOSENSORS AND BIOELECTRONICS, vol. 42, 7 November 2012 (2012-11-07), pages 219-224, XP055304700, NL ISSN: 0956-5663, DOI: 10.1016/j.bios.2012.10.094 the whole document ----- -/--	1-23



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

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"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

28 September 2016

Date of mailing of the international search report

10/10/2016

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INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2016/050246

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HEERING H A ET AL: "Direct detection and measurement of electron relays in a multicentered enzyme: voltammetry of electrode-surface films of E. coli fumarate, an iron-sulfur flavoprotein", JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, AMERICAN CHEMICAL SOCIETY, US, vol. 119, 1 January 1997 (1997-01-01), pages 11628-11638, XP002193725, ISSN: 0002-7863, DOI: 10.1021/JA9723242 page 11630, left-hand column, paragraph 2 -----	1-23
Y	K. L. TURNER ET AL: "Redox Properties of Flavocytochrome c 3 from Shewanella frigidimarina NCIMB400 +", BIOCHEMISTRY, vol. 38, no. 11, 1 March 1999 (1999-03-01), pages 3302-3309, XP55305041, US ISSN: 0006-2960, DOI: 10.1021/bi9826308 page 3303, left-hand column, paragraph 3 - paragraph 4 -----	1-23
Y	WO 03/019170 A1 (YISSUM RES DEV CO [IL]; WILLNER ITAMAR [IL]; KATZ EVGENY [IL]) 6 March 2003 (2003-03-06) claims 1-19; figures 3,7 -----	1-23
X,P	S. SHIPOVSKOV ET AL: "Electrocatalytic interconversion of NADH and NAD + by Escherichia coli flavohemoglobin", CHEMICAL COMMUNICATIONS - CHEMCOM., vol. 51, no. 89, 1 November 2015 (2015-11-01), pages 16096-16098, XP055304694, ISSN: 1359-7345, DOI: 10.1039/C5CC06317E the whole document -----	1-23

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/DK2016/050246

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 03019170	A1	06-03-2003	
		EP 1421370 A1	26-05-2004
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