PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

GEYRA GASSNER KESTEN
55 Yigal Alon St.
Tel Aviv 6789115
Israel

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)*	28 Oct 2018
Applicant's or agent's file reference BGU-P-76-PCT	**FOR FURTHER ACTION** See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IL2018/050863	02 Aug 2018	02 Aug 2017

International Patent Classification (IPC) or both national classification and IPC
IPC (2018.01) C07K 19/00 C12N 15/62 C12Q 1/32 G01N 33/66

Applicant
B. G. NEGEV TECHNOLOGIES AND APPLICATIONS LTD., AT BEN-GURION UNIVERSITY

1. This opinion contains indications relating to the following items:

- [x] Box No. I Basis of the opinion
- [ ] Box No. II Priority
- [ ] Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- [ ] Box No. IV Lack of unity of invention
- [x] Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- [ ] Box No. VI Certain documents cited
- [ ] Box No. VII Certain defects in the international application
- [x] Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA:	Date of completion of this opinion	Authorized officer
Israel Patent Office Technology Park, Bldg.5, Malcha, Jerusalem, 9695101, Israel	17 Oct 2018	MAZEL Alexander
Facsimile No. 972-2-5651616		Telephone No. 972-2-5651716

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Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   [X] the international application in the language in which it was filed.

   [ ] a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. [ ] This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

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Box No. V **Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	1-44	YES
	Claims		NO
Inventive step (IS)	Claims		YES
	Claims	1-44	NO
Industrial applicability (IA)	Claims	1-44	YES
	Claims		NO

2. Citations and explanations:

2.1 Reference is made to the following documents:

D1: US2017121751 (ARKRAY, Inc, ; Ultizyme International Ltd), 04 May 2017 (2017/05/04).

D2: WO2017094776 08 (KIKKOMAN CORP), June 2017 (2017/06/08).

D3: Yamaoka, H., Yamashita, Y., Ferri, S., & Sode, K. (2008). Site directed mutagenesis studies of FAD-dependent glucose dehydrogenase catalytic subunit of Burkholderia cepacia. *Biotechnology letters*, *30*(11), 1967-1972.

D4: Simone, A. D. (2016). Engineering the genetic code of Escherichia coli with methionine analogues and bioorthogonal amino acids for protein immobilization.

2.2 The present claim 1 relates to a recombinant protein, comprising: (a) alpha subunit of an FAD-GDH; and (b) a minimal c-type cytochrome peptide.

The present independent claim 23 relates to an electrode coupled to a recombinant protein, the recombinant protein comprising:
(a) a cofactor of a redox enzyme;
(b) a redox enzyme;
(c) a linker moiety configured to link any one of: the cofactor or the enzyme to an electron transfer (ET) domain; and
(d) an ET domain configured to transfer electrons between the electrode and the cofactor;
wherein a distance between said ET domain and the electrode is in the range of 0 to 14 Å.

The present independent claim 37 relates to a method for determining an analyte in a liquid medium, the analyte being capable to undergo a biocatalytic oxidation or reduction reaction in the presence of an oxidizer or a reducer, respectively, the method comprising:
(i) providing the disclosed device in an embodiment thereof;
(ii) contacting the device with the liquid medium;
(iii) measuring the electric signal generated between the cathode and the anode, the electric signal being indicative of the presence and/or the concentration of the analyte; and
(iv) determining the analyte based on the electric signal.

Additional claims provide technical details concerning said recombinant protein, said electrode

and said method.

2.3 Novelty

The subject-matter of claims 1-44 is novel and therefore complies with PCT Article 33(2).

2.4 Inventive step

2.4.1 D1 discloses using of FAD-GDH complex obtained or derived from Burkholderia cepanian for preparing an electrode for measurement of glucose. According to D1 (paragraphs [0006-0008], [0082-0093], and [101]), said complex comprises alpha subunit (mutant GDH) and beta subunit (cytochrome c). According to D1 (paragraphs [0008], [0084], [0098-0100], [0112-0114]) said alpha subunit acts on glucose and is coupled (linked) to an electrode via said beta subunit acting as an electron transfer unit. According to paragraph [0098] of D1 the electrode is gold or carbon electrode and is part of glucose sensor (i.e. device). According to paragraph [0012] of D1, the measurement of glucose is based on measurement of decoloring of 2, 6-Dichlorophenolindophenol reporter (decreasing of light absorption at 600nm) as a result of reduction of said reporter in course of reaction between said FAD-GDH complex and glucose. D1 also discloses DNA encoding said subunits and cells and expression vectors comprising said DNA. The difference between D1 and the present claims 1, 13-15, 17-21, 23-26 and 28-44 is that D1 does not teach about fusion recombinant protein comprising alpha subunit of GDH-FAD and minimal cytochrome peptide. However, the skilled person would compliment said trait from D2.

2.4.2 D2 (whole document, especially claims 1-14) relates to a fusion protein comprising GDH and cytochrome moiety linked by linker (see for example paragraphs [0064-0065 of D2). Said fusion protein is part of glucose sensor and transfers electrons directly to an electrode in course of glucose measurements. According claim 4 of D2, FAD-GDH is from Mucor prainii. According to D2 (page 5, third paragraph) the cytochrome moiety is full or partial cytochrome domain thermophilic bacteria cellobiose dehydrogenase. In light of paragraph 6 of the priority document and owing to lack of clear definition of the term "minimal cytochrome peptide", it is impossible to distinguish cytochrome moiety of D2 from minimal cytochrome peptide of the present application.

Advantages of D2 allowing direct transfer of electrons from the fusion protein that is immobilized on electrode to electrode surface (see for example [0132], [0135] of D2) in comparison to D1 requiring assembly of separate FAD-GDH and cytochrome on electrode surface before electron transfer became possible are clear to the skilled person. Therefore, the skilled person has motivation to use D2 to modify the approach of D1. Thus, the combination D1-D2 takes away inventiveness of the present claims 1, 13-15, 17-21, 23-26 and 28-44.

2.4.3 Additional difference between D1 and the present claims 8-10 and 11 is that D1 does not explicitly disclose FAD-GDH-cytochrome c complex comprises a porhyrin having bi- or tri-valent metal and that said complex has peroxidase/oxidase activity. However heme (i.e. porphyrin) domain with iron atom is an integral part of the cytochrome moiety of D2 (see [0032] and [0052]). Also, according paragraphs [0004-0006] of D2, GDH is an oxidoreductase having measurable peroxidase activity. Thus, the combination of D1 and D2 takes away also inventiveness of the present claims 8-10 and 11.

2.4.4 In light of combination D1 and D2 it is obvious to the skilled person that different FAD-

GDH and minimal cytochrome peptides can be successfully used for the present invention. Therefore, in absence of indications at advantages conferred by specific FAD-GDH, minimal cytochrome peptides and DNAs set forth in the present claims 5, 6, 12 and 16, the inventiveness of said claims cannot be acknowledged.

2.4.5 In course of the modification of the approach of D1 according principles of D2 (see the section 2.4.2 above) the skilled person would retain using of a specific alpha subunit from Burkholderia cepacian as teaches D1. Indeed, according to D3 (see abstract, pages 1967-1968) said enzyme has high enzymatic activity and is resistant to oxidation and heat. Thus, the combination D1-D3 takes away inventiveness of the present claims 3 and 4.

2.4.6 Additional difference between D1 and the present claims 22, 32 and 33 is that D1 do not teach coupling to an electrode via non-canonical amino acid (ncAA) residue. However, the skilled person would compliment this trait from D4 . D4 (pages 73-74, figs. 47 and 48) describes using "click" chemistry for immobilization of lipase from Thermoanaerobacter thermohydrosulfuricus (TTL) to azide-agarose beads via ncAA Propargyl-lysine incorporated into said lipase. Moreover, the similar approach was used for immobilizing of enzymes (such as laccase) on an electrode via ncAA. It is obvious to the skilled person that the approach of D4 allows fast, simple and efficient way for immobilization of proteins to the surfaces, including immobilizing enzymes on electrodes. Therefore, the skilled person has incentive to use D4 in order to improve the inventions set forth in D1 and D2. Thus, combination of D1, D2 and D4 takes away inventiveness of the present claims 22, 32 and 33.

2.4.7 The additional features of the dependent claims 2 and 27 do not confer an inventive step to said claim because said features are trivial and obvious to the skilled person.

2.4.8 Thus, the subject-matter of claims 1-44 does not involve an inventive step and therefore does not comply with PCT Article 33(3).

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Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

1. The scope of the present claim 1 is not clear owing to lack of clear and general definition of the term "minimal cytochrome peptide". Thus, said claim 1 is not clear, in contravention to requirement of Article 6 PCT.

2. The abbreviation "FAD-GDH" in claim 1 should be spelled in full for the sake of the clarity required by Article 6 PCT.

3. The present claim 24 is unclear owing lack of sufficient clarity of the expression "recombinant protein in the form of formula (a)-(b)-(c)-(d)". Also, it seems that in the present formulation said claim 24 is redundant. Thus, said claim 24 is not clear and cause lack of conciseness to the present set of claims, in contravention to requirements of Article 6 PCT.

4. The present claim 37 is unclear owing lack of clarity of the expression "the disclosed device in an embodiment thereof". Thus, said claim 37 is not clear, in contravention to requirement of Article 6 PCT.