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(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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25 June 2015

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(54) **Title**: MODIFIED ALPHA HEMOLYSIN POLYPEPTIDES AND METHODS OF USE

(57) **Abstract**: Provided herein are alpha hemolysin polypeptides comprising modified amino acid sequences that can reduce the rate of translocation of a polymer. Also provided herein are apparatuses and devices comprising modified hemolysin polypeptides. Also provided herein are methods of using modified alpha hemolysin proteins for use in characterizing and/or sequencing a polymer or for use as molecular sensors.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 13/76698

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07K 1/00, G01N 33/48 (2014.01)
USPC - 530/350, 702/20
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)
USPC- 530/350, 702/20

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC- 435/6.1; 204/520, 204/540, 536/23.7

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(PGPB,USPT,USOC,EPAB,JPAB), Google/Scholar: Alpha hemolysin, alpha.HL, nanopore, translocation, sequencing, substitution, position, mutation
GenCore 6.4.1: SEQ ID NO: 1, 2

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/102120 A1 (Sanghera, et al.) 28 August 2008 (28.08.2008) SEQ ID NO 2	1 and 2
X	WO 2009/077734 A2 (Clarke, et al.) 25 June 2009 (25.06.2009) SEQ ID NO 2	1 and 2
X	US 2010/0203024 A1 (Terman, et al.) 12 August 2010 (12.08.2010) SEQ ID NO 63	1 and 2
X,P	WO 2012/178097 A1 (Barrall, et al.) 27 December 2012 (27.12.2012) Example 1, SEQ ID NO: 1	1

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"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	Date of mailing of the international search report
22 March 2014 (22.03.2014)	21 MAY 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 13/76698

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.:
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:

3. ☒ Claims Nos.: 4-58, 61, 62
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I+: claims 1-3, 59, and 60, drawn to a modified alpha hemolysin polypeptide comprising a modified alpha hemolysin amino acid sequence, wherein: the modified alpha hemolysin amino acid sequence comprises one or more amino acid substitutions at one or more positions corresponding to positions 1-109 and 149-293 of SEQ ID NO: 1; and at least one of the one or more amino acid substitutions independently is to a non-native amino acid, wherein the non-native amino acid is hydrophobic or aromatic, or hydrophobic and aromatic. Group I+ will be searched to the extent that it reads on a substitution at the position 1. It is believed that claims 1 and 2 read on this first named invention. Applicants must indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.
An exemplary election would be: a substitution at the position 105, i.e. claims 1-3.

********** See Supplemental Sheet to continue **********

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1 and 2, restricted to a substitution at the position 1

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

********** Supplemental Sheet **********

In Continuation of Box III. Observations where unity of invention is lacking:

Group II+: claims 63-70, drawn to a method of sequencing a polymer with a modified alpha hemolysin polypeptide. Group II+ will be searched upon payment of additional fee(s). An exemplary election would be: a substitution at the position 1. It is believed that claims 63 -66 and 70 (in part) read on this named invention. Applicants must indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.
Another exemplary election would be: a substitution at the position 105, i.e. claims 63-67 and 70 (in part).

Group III+: claims 71-88, drawn to a method of translocating a polymer through a modified alpha hemolysin polypeptide comprising: (b) determining a translocation time of the polymer through the modified alpha hemolysin polypeptide. Group III+ will be searched upon payment of additional fee(s). An exemplary election would be: a substitution at the position 1. It is believed that claims 71-75, 79 (in part), 80-84, 87, and 88 (in part) read on this named invention. Applicants must indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.
Another exemplary election would be: a substitution at the position 105, i.e. claims 71-76, 79 (in part), 80-85, 87, and 88 (in part).

Group IV+: claims 89-97, drawn to a nanopore device comprising a modified alpha hemolysin protein. Group IV+ will be searched upon payment of additional fee(s). An exemplary election would be: a substitution at the position 1. It is believed that claims 89-93 and 97 (in part) read on this named invention. Applicants must indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.
Another exemplary election would be: a substitution at the position 105, i.e. claims 89-94 and 97 (in part).

The inventions listed as Groups I+ through IV+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features
The special technical feature of each invention of Groups I+ through IV+ is a specific substitution(s) in the amino acid sequence SEQ ID NO:1.

The inventions of Group I+ do not include the shared or common technical feature of an alpha-hemolysin nanopore device or a method of using said device, as required by Groups II+ through IV+.

The inventions of Groups IV+ do not include the shared or common technical feature of a method of using an alpha-hemolysin nanopore device, as required by Groups II+ and III+.

The inventions of Group II+ do not include the shared or common technical feature of a method comprising (b) determining a translocation time of the polymer through the modified alpha hemolysin polypeptide, as required by Group III+.

The inventions of Group III+ do not include the shared or common technical feature of a method comprising (b) determining the sequence of the polymer according to one or more electrical changes across or through the modified alpha hemolysis protein pore, as required by Group II+.

Common Technical Features
The inventions of Group I+ share the technical feature of a modified alpha hemolysin polypeptide of claim 1. However, this shared technical feature does not represent a contribution over prior art that would otherwise unify the groups, because it is anticipated by US 7,608,276 B2 to Masignani, et al. (27 October 2009) (hereinafter " Masignani") disclosing a modified alpha hemolysin polypeptide comprising one or more amino acid substitutions at one or more positions corresponding to positions 1-109 and 149-293 of SEQ ID NO: 1; and the substitution is to a non-native hydrophobic amino acid (SEQ ID NO 1096, with on substitution corresponding to the position 274 of the claimed SEQ ID NO: 1, specifically Thr274Ile).

The inventions of Group II+ share the technical feature of a method of claim 63. However, this shared technical feature does not represent a contribution over prior art that would otherwise unify the groups, because it is anticipated by a paper titled "Continuous base identification for single-molecule nanopore DNA sequencing" by Clarke, et al. (Nat Nanotechnol. 2009, 4(4):265-70) (hereinafter "Clarke") disclosing a method of sequencing a polymer (Abstract, "A single-molecule method for sequencing DNA... we show that a protein nanopore with a covalently attached adapter molecule can continuously identify unlabelled nucleoside 5'-monophosphate molecules with accuracies averaging 99.8%. Methylated cytosine can also be distinguished from the four standard DNA bases: guanine, adenine, thymine and cytosine. The operating conditions are compatible with the exonuclease, and the kinetic data show that the nucleotides have a high probability of translocation through the nanopore and, therefore, of not being registered twice. This highly accurate tool is suitable for integration into a system for sequencing nucleic acids and for analysing epigenetic modifications") with a modified alpha hemolysin polypeptide (pg 266, Fig 1 and its legend, "Structures of haemolysin mutants. a, Structure of the WT-(M113R/N139Q)6(M113R/N139Q/L135C)1 mutant... b, Close-up of the b barrel showing the arginines at position 113 and the location of the cysteines in the mutants tested in this study") comprising:
(a) contacting a polymer with a modified alpha hemolysin polypeptide, wherein the modified alpha hemolysin polypeptide comprises an amino acid sequence of a reference alpha hemolysin protein comprising one or more amino acid substitutions (pg 266, Fig 2 and its legend, "Single-channel recordings comparing permanent and transient adapters. a, The WT-(M113R/N139Q)7 aHL pore showing transient adapter binding ... and nucleotide detection. Histogram of the residual current of nucleotide binding events. b, Corresponding data for the WT-(M113R/N139Q)6(M113R/N139Q/L135C)1 mutant..., allowing continuous nucleotide detection and enhanced nucleotide discrimination (see histogram)"), and

********** See the following Supplemental Sheet to continue **********

********** Supplemental Sheet **********

In Continuation of the Preceding Supplemental Sheet:

(b) determining the sequence of the polymer according to one or more electrical changes across or through the modified alpha hemolysis protein pore (pg 267, Fig 3 and its legend, "Nucleotide event distributions with the permanent adapter. a, Single-channel recording from the WT-(M113R/N139Q)6(M113R/N139Q/L135C)1-am6amDP1bCD pore showing dGMP, dTMP, dAMP and dCMP discrimination").

The inventions of Group IV+ share the technical feature of a nanopore device of claim 89. However, this shared technical feature does not represent a contribution over prior art that would otherwise unify the groups, because it is anticipated by a paper titled "Individual RNA base recognition in immobilized oligonucleotides using a protein nanopore" by Ayub, et al. (Nano Lett. ePub 19 October 2012; 12(11):5637-43) (hereinafter "Ayub") disclosing
a nanopore device (Supplemental Materials, pg 1, 2, recording chamber, "The [alpha] HL pores and the oligonucleotides (Table S1) were added to the cis compartment") comprising a modified alpha hemolysin protein (pg 3 [of the uploaded document], 3rd para, "We examined the ability of WT, NN and NNY [alpha]HL pores to distinguish between rG, rA, rU, and rC within individual nucleic strands").

The inventions of Group III+ share the technical feature of a method of claims 71 and 80. However, this shared technical feature does not represent a contribution over prior art that would otherwise unify the groups, because it is obvious over US 2012/0310543 A1 to Hibbs, et al. (06 December 2012) (hereinafter "Hibbs") in view of an article titled "Nucleobase recognition in ssDNA at the central constriction of the alpha-hemolysin pore" by Stoddart, et al. (Nano Lett. 2010, 10(9):3633-3637) (hereinafter "Stoddart") as follows:

Hibbs discloses a method of translocating a polymer (para [0026], "measurement device 1 controls the translocation of a polymer 18 through orifice 17 utilizing a translocation means or means for controlling the velocity of a polymer through orifice 17 in the form of a power source 20") through a modified alpha hemolysin polypeptide (para [0029], "it has been determined that the rate of passage of DNA through an .alpha.HL protein pore can be reduced by orders of magnitude by... mutating .alpha.HL or adding an internal adapter to reduce its internal dimensions will increase the energy barrier, E, resulting in a reduction in the diffusion rate, D"; para [0037], "orifice 17 is preferably a protein pore mutated or chemically altered to increase the effective friction of polymer 18 through orifice 17"; claim 8, "the nanopore is a mutated biological protein pore") comprising:
(a) contacting a polymer with a modified alpha hemolysin polypeptide, under conditions in which the polymer translocates through the modified alpha hemolysin polypeptide (para [0026, "In general, measurement device 1 controls the translocation of a polymer 18 through orifice 17 utilizing a translocation means or means for controlling the velocity of a polymer through orifice 17 in the form of a power source 20"; para [0028], "Experiments have shown that DNA passage through a nano-scale orifice of comparable diameter to the DNA is limited by an essentially frictional interaction, such that the average translocation velocity, v.sub.DC, is proportional to the applied force. Because each base of DNA carries a net charge, a force to induce translocation through a pore can easily be applied by imposing an electric field across the pore"), and
(b) determining a translocation time of the polymer through the modified alpha hemolysin polypeptide (claim 26, "establishing an initial measurement time based on properties of the nanopore sensing system; calculating an initial translocation velocity of the polymer in the nanopore sensing system based on the initial measurement time; deriving a relationship between the sequencing order error rate and the monomer identification error rate; and selecting a final measurement time and a final translocation velocity").
Hibbs does not disclose that the modified alpha hemolysin polypeptide comprises an amino acid sequence of a reference alpha hemolysin protein comprising one or more amino acid substitutions.
This limitation is disclosed by Stoddart: "The a-hemolysin (aHL) protein nanopore contains three recognition points capable of nucleobase discrimination in individual immobilized ssDNA molecules. We have modified the recognition point R1 by extensive mutagenesis of residue 113. Amino acids that provide an energy barrier to ion flow (e.g. bulky or hydrophobic residues) strengthen base identification, while amino acids that lower the barrier weaken it. Amino acids with related side chains produce similar patterns of nucleobase recognition providing a rationale for the redesign of recognition points" (Abstract).
It would have been obvious to one of ordinary skill in the art at the time of the invention to combine Hibbs and Stoddart by using the modified a-hemolysin of Stoddart (pg 4 [of the posted document], para 2 and 3, "amino acids with small or uncharged hydrophilic side chains discriminate between bases poorly, Gly not at all. By contrast, amino acids with hydrophobic, basic or aromatic side chains show superior base discrimination, which varies in character between the amino acid classes: for the hydrophobic amino acids (Val, Leu, Ile): A < G < C < T (order of IRES value); basic amino acids (Lys, Arg): G < T < A . C; aromatic amino acids (Tyr, Trp): T <G < A < C") in the translocation method of Hibbs, because Hibbs specifically discloses that translocation of DNA through an .alpha.HL protein pore can be controlled by mutating .alpha.HL (para [0029], "it has been determined that the rate of passage of DNA through an .alpha.HL protein pore can be reduced by orders of magnitude by... mutating .alpha.HL... to reduce its internal dimensions will increase the energy barrier, E, resulting in a reduction in the diffusion rate, D").

In addition, SEQ ID NO: 1 was known in the art at the time of the invention, as evidenced by US 2009/0167288 A1 to Reid, et al. (02 July 2009) (hereinafter "Reid") disclosing the amino acid sequence 100% identical to the claimed SEQ ID NO: 1 (SEQ ID NO 3).

Therefore, Groups I+ through IV+ lack unity under PCT Rule 13.