differences between nucleotides could be improved further. Accordingly, there is a need to identify novel ways to improve nanopore sensing features.

SUMMARY

The disclosure relates to modified CsgF peptides, in particular truncated CsgF fragments, binding the CsgG pore and thereby introducing another additional channel or pore constriction within the CsgG pore. Other examples of the disclosure also relate to an isolated transmembrane pore complex, and the use of said CsgG:CsgF complexes and modified CsgF peptides or fragments in a nanopore sensing platform with two consecutive reader heads.

In one aspect, the present disclosure relates to a truncated CsgF peptide that lacks the C-terminal head domain of CsgF and at least part of the neck domain of CsgF, and comprises a CsgG-binding region, and a region that forms a constriction in a pore comprising CsgG and the truncated CsgF peptide, wherein the neck domain comprises amino acid residues from amino acids 36-60 of SEQ ID NO: 6; the head domain is the region C-terminal to the neck domain; the CsgG binding region comprises amino acid residues 1 to 8 and/or 29 to 32 of the SEQ ID NO: 6; and the region that forms a constriction in the pore comprises amino acid residues 9 to 28 of SEQ ID NO: 6. In another aspect, the present disclosure relates to a polynucleotide which encodes a CsgF peptide as disclosed herein. In another aspect, the present disclosure relates to a pore comprising a CsgG pore and a truncated CsgF peptide as disclosed herein, wherein the truncated CsgF peptide is bound to CsgG and forms a constriction in the pore.

In another aspect, the present disclosure relates to a method for producing a pore as described herein, the method comprising: a. co-expressing one or more CsgG monomers and a truncated CsgF peptide as disclosed herein in a host cell, thereby allowing transmembrane pore complex formation in the cell; or b. contacting one or more purified CsgG monomers with a CsgF peptide as disclosed herein, thereby allowing *in vitro* formation of the pore. In yet another aspect, the present disclosure relates to a method for determining the presence, absence or one or more characteristics of a target analyte, comprising the steps of: (i) contacting the target analyte with a pore as described herein, such that the target analyte moves into the pore complex; and (ii) taking one or more measurements as the analyte moves through the pore complex and thereby determining the presence, absence or one or more characteristics of the analyte. In yet another aspect, the present disclosure relates to a method of characterising a polynucleotide or a (poly)peptide using a pore as described herein.

In yet another aspect, the present disclosure relates to the use of the pore as described herein to determine the presence, absence or one or more characteristics of a target analyte. In yet another aspect, the present disclosure relates to a kit for characterising a target analyte comprising (a) pore as described herein and (b) the components of a membrane.

The first example of the disclosure relates to a pore comprising a CsgG pore and a CsgF peptide. In one example, the CsgF peptide comprises a CsgG-binding region, and a region that forms a constriction in the pore. In one example the CsgF peptide is a truncated CsgF peptide lacking the C-terminal head domain of CsgF. In another example the CsgF peptide is a truncated CsgF peptide lacking the C-terminal head and a portion of the neck domain of CsgF. In another example the CsgF peptide is a truncated CsgF peptide lacking the C-terminal head and neck domains of CsgF. The pore is also referred to herein as a pore complex and as an isolated pore complex. The isolated pore complex comprising a CsgG pore, or a homologue or mutant thereof, and a modified CsgF peptide, or a homologue or mutant thereof, in particular truncated CsgF fragments, or homologues or mutants thereof. In one example, said modified CsgF peptide, or homologues or mutants, is located in the lumen

CLAIMS

1. A truncated CsgF peptide that lacks the C-terminal head domain of CsgF and at least part of the neck domain of CsgF, and comprises a CsgG-binding region, and a region that forms a constriction in a pore comprising CsgG and the truncated CsgF peptide, wherein

the neck domain comprises amino acid residues from amino acids 36-60 of SEQ ID NO: 6;

the head domain is the region C-terminal to the neck domain;

the CsgG binding region comprises amino acid residues 1 to 8 and/or 29 to 32 of the SEQ ID NO: 6; and

the region that forms a constriction in the pore comprises amino acid residues 9 to 28 of SEQ ID NO: 6.

2. The CsgF peptide according to claim 1, which has a length of from 25 to 50 amino acids.

3. A CsgF peptide according to any claim 1 or claim 2, which comprises:

a. the amino acid sequence from residue 1 of SEQ ID NO: 6 up to any one of residues 28 to 45 of SEQ ID NO: 6, or a homologue or variant thereof with at least 80% sequence identity to SEQ ID NO: 6; and/or

b. SEQ ID NO: 39 (residues 1 to 29 of SEQ ID NO: 6), or a homologue or variant thereof with at least 80% sequence identity to SEQ ID NO: 6; or

c. SEQ ID NO: 15 (residues 1 to 34 of SEQ ID NO: 6) or a homologue or variant thereof with at least 80% sequence identity to SEQ ID NO: 6, SEQ ID NO: 54 (residues 1 to 30 of SEQ ID NO: 6) or a homologue or variant thereof with at least 80% sequence identity to SEQ ID NO: 6, SEQ ID NO: 40 (residues 1 to 45 of SEQ ID NO: 6) or a homologue or variant thereof with at least 80% sequence identity to SEQ ID NO: 6, or SEQ ID NO: 55 (residues 1 to 35 of SEQ ID NO: 6) or a homologue or variant thereof with at least 80% sequence identity to SEQ ID NO: 6.

4. A CsgF peptide according to claim 3, wherein:

a. one or more residues in the region between residues 1 and 28 to 45 of SEQ ID NO: 6, SEQ ID NO: 15, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 54, or SEQ ID NO: 55 (residues 1 to 35) are modified, and optionally

the peptide comprises a modification at one or more of the following positions: G1, T4, F5, R8, N9, N11, F12, A26 and Q29 and

the modification is the introduction of a cysteine, a hydrophobic amino acid, a charged amino acid, a non-native reactive amino acid, or photoreactive amino acid; and/or

b. the peptide comprises a modification at one or more of the following positions: N15, N17, A20, N24, A28 and D34 of SEQ ID NO: 6, and optionally

the peptide comprises one or more of the substitutions: N15S/A/T/Q/G/L/V/I/F/Y/W/R/K/D/C, N17S/A/T/Q/G/L/V/I/F/Y/W/R/K/D/C, A20S/T/Q/N/G/L/V/I/F/Y/W/R/K/D/C, N24S/T/Q/A/G/L/V/I/F/Y/W/R/K/D/C, A28S/T/Q/N/G/L/V/I/F/Y/W/R/K/D/C, and D34F/Y/W/R/K/N/Q/C of SEQ ID NO: 6; and/or

c. the peptide comprises one or more of the following substitutions: G1C, T4C, N17S, and D34Y or D34N of SEQ ID NO: 6.

5. A CsgF peptide according to any one of claims 1 to 4, which further comprises:

a. all or part of an enzyme cleavage site at the C-terminal end; and/or

b. a signal peptide.

6. A polynucleotide which encodes a CsgF peptide according to any one of claims 1 to 5.

7. A pore comprising a CsgG pore and a truncated CsgF peptide according to any one of claims 1 to 6, wherein the truncated CsgF peptide is bound to CsgG and forms a constriction in the pore.

8. The pore according to claim 7, wherein:

a. the truncated CsgF peptide is inserted into the lumen of the CsgG pore; and/or

b. the CsgG pore comprises from 6 to 10 CsgG monomers; and/or

c. the ratio of CsgG monomers to truncated CsgF peptides in the pore is 1:1.

9. The pore according to claim 7 or 8, wherein the CsgF peptide and the CsgG pore are covalently coupled and wherein:

I. the covalent coupling is via:

(i) a cysteine residue at a position corresponding to 132, 133, 136, 138, 140, 142, 144, 145, 147, 149, 151, 153, 155, 183, 185, 187, 189, 191, 201, 203, 205, 207 or 209 of SEQ ID NO: 3, or of a homologue thereof;

(ii) a non-native reactive or photoreactive amino acid at a position corresponding to 132, 133, 136, 138, 140, 142, 144, 145, 147, 149, 151, 153, 155, 183, 185, 187, 189, 191, 201, 203, 205, 207 or 209 of SEQ ID NO: 3, or of a homologue thereof; and/or

II. the CsgF peptide and the CsgG pore are covalently linked via residues at a position corresponding to one or more of the following pairs of positions of SEQ ID NO: 6 and SEQ ID NO: 3, respectively: 1 and 153, 4 and 133, 5 and 136, 8 and 187, 8 and 203, 9 and 203, 11 and 142, 11 and 201, 12 and 149, 12 and 203, 26 and 191, and 29 and 144; and/or

III. the covalent linkage is a disulphide bond, or click chemistry.

10. The pore according to any one of claims 7 to 9, wherein:

I. the interaction between the CsgF peptide and the CsgG pore is stabilised by hydrophobic interactions, or electrostatic or covalent interactions at a position corresponding to one or more of the following pairs of positions of SEQ ID NO: 6 and SEQ ID NO: 3, respectively: 1 and 153, 4 and 133, 5 and 136, 8 and 187, 8 and 203, 9 and 203, 11 and 142, 11 and 201, 12 and 149, 12 and 203, 26 and 191, and 29 and 144; and/or

II. the CsgG pore comprises at least one monomer comprising one or more modification corresponding to the following modifications in SEQ ID NO:3:

(i) a modification at one or more positions Y51, N55 and F56;

(ii) at least one substitution selected from R97W or R97Y and R93W or R93Y;

(iii) deletion of V105, A106 and I107 of SEQ ID NO:3;

(iv) deletion of one or more of positions R192, F193, I194, D195, Y196, Q197, R198, L199 and E201 of SEQ ID NO:3;

(v) at least one substitution selected from K94N/Q/R/F/Y/W/L/S, D43S, E44S, F48S/N/Q/Y/W/I/V/H/R/K, Q87N/R/K, N91K/R, R97F/Y/W/V/I/K/S/Q/H, E101I/L/A/H, N102K/Q/L/I/V/S/H, R110F/G/N, Q114R/K, R142Q/S, T150Y/A/V/L/S/Q/N; and/or

(vi) a modification at one or more of positions I41, R93, A98, Q100, G103, T104, A106, I107, N108, L113, S115, T117, Y130, K135, E170, S208, D233, D238, E244, Q42, E44, L90, N91, I95, A99, E101 and Q114.

11. The pore according to claim 10, wherein the CsgG pore comprises at least one monomer comprising one or more modification corresponding to the following modifications in SEQ ID NO:3:

(i) at least one substitution selected from Y51A/I/V/S/T, N55A/I/V/S/T and F56/A/I/V/S/T/Q;

(ii) the substitution R97W;

(iii) deletion of F193, I194, D195, Y196, Q197, R198 and L199 or deletion of D195, Y196, Q197, R198 and L199;

(iv) deletion of V105, A106 and I107;

(v) a substitution selected from K94Q and K94N;

(vi) at least one substitution selected from Q42K or Q42R; E44N or E44Q; L90R or L90K; N91R or N91K; I95R or I95K; A99R or A99K; E101H, E101K, E101N, E101Q or E101T; and/or Q114K; and/or

(vii) the substitution N55V.

12. The pore of any one of claims 7 to 11, wherein:

a. the CsgG pore comprises at least one monomer that comprises R or K at a position corresponding to R192 of SEQ ID NO:3; and/or

b. the pore is a double pore comprising two CsgG pores, wherein the CsgF peptide is inserted into the lumen of at least one of the CsgG pores.

13. A pore according to any one of claims 7 to 12, which is comprised in a membrane.

14. A method for producing a pore according to any one of claims 7 to 13, the method comprising:

a. co-expressing one or more CsgG monomers and a truncated CsgF peptide according to any one of claims 1 to 6 in a host cell, thereby allowing transmembrane pore complex formation in the cell; or

b. contacting one or more purified CsgG monomers with a CsgF peptide according to any one of claims 1 to 6, thereby allowing *in vitro* formation of the pore.

15. A method for producing a pore according to claim 14, wherein the method comprises expressing in a host cell a CsgF peptide according to any one of claims 1 to 6 comprising an enzyme cleavage site positioned such that cleavage of the CsgF peptide produces a truncated CsgF peptide according to any one of claims 1 to 6, and cleaving the CsgF peptide.

16. The method according to claim 14 or 15, wherein the modified CsgF peptide comprises SEQ ID NO:15 or SEQ ID NO:16, or a homologue or mutant thereof with at least 80% sequence identiy to SEQ ID NO: 15 or SEQ ID NO: 16.

17. A method for determining the presence, absence or one or more characteristics of a target analyte, comprising the steps of:

(i) contacting the target analyte with a pore according to any one of claims 7 to 13, such that the target analyte moves into the pore complex; and

(ii) taking one or more measurements as the analyte moves through the pore complex and thereby determining the presence, absence or one or more characteristics of the analyte.

18. A method according to claim 17, wherein:

the analyte is a polynucleotide, a (poly)peptide, a polysaccharide, a small organic or inorganic compound, such as pharmacologically active compounds, toxic compounds and pollutants.

19. A method according to claim 18, wherein the analyte is a polynucleotide and:

a. the polynucleotide comprises at least one homopolymeric region; and/or

b. the method comprises determining one or more characteristics selected from (i) the length of the polynucleotide, (ii) the identity of the polynucleotide, (iii) the sequence of the polynucleotide, (iv) the secondary structure of the polynucleotide and (v) whether or not the polynucleotide is modified.

20. A method of characterising a polynucleotide or a (poly)peptide using a pore according to any one of claims 7 to 13.

21. Use of the pore according to any one of claims 7 to 13 to determine the presence, absence or one or more characteristics of a target analyte.

22. A kit for characterising a target analyte comprising (a) pore according to any one of claims 7 to 13 and (b) the components of a membrane.