engineered proteins are derived from libraries of variants wherein exposed surface residues are diversified upon a core scaffold[38-40].

Betton et al (1997)[17] reports fusing β lactamase into several accommodating permissive sites in maltose binding proteins using 9-10 amino acid linkers (DPGG-insert-YPGDP (SEQ ID NOS: 3, 4) and PDPGG–insert-YPGGP (SEQ ID NOS: 5, 6). Collinet[18] reports inserting β lactamase or dihydrofolate reductase into permissive sites of phosphoglycerate kinase using amino acid residues SGG or GG at the N terminal insertion point and GAG at the C terminal insertion point. Studies of the enzyme β-lactamase, which confers bacterial resistance to ampicillin, have also revealed potential sites into which peptide insertions could be accommodated[19]. Vandevenne et al reports the insertion into one of these sites of a chitin binding domain protein of the human macrophage chitotriosidase[20], using a construct that introduced 6-7 amino acids each at the N and C termini of the insertion. Crasson et al[21] have demonstrated functionalization of a nanobody by inserting it into a permissive site of β lactamase joining two helices.

Summary

The present inventors have found that the incorporation of an entire binding domain ("donor diversity scaffold domain") within a second binding domain ("a recipient scaffold domain") increases the diversity of populations of binding members and allows the selection and isolation of binding members with advantageous properties from these populations. This may be useful for example in selecting binding members which bind to target molecules, such as ion channels, that are difficult to target with conventional antibodies.

In one aspect, the present disclosure refers to a method of screening comprising;

(i) providing a founder library of binding members,

each binding member in the library comprising a fusion protein and optionally, a partner domain associated with the fusion protein,

wherein the fusion protein comprises a donor diversity scaffold domain inserted within a recipient diversity scaffold domain, optionally wherein the N and C terminals of the donor diversity scaffold domain are linked to the recipient diversity scaffold domain with linkers of up to 4 amino acids,

wherein the donor diversity scaffold domain is a cysteine-rich peptide consisting of 100 or fewer amino acids, comprising two or more disulphide bridges formed by four or more cysteine residues within said donor diversity scaffold domain, wherein the cysteine-rich peptide is an ion channel modulating peptide, a venom peptide or a knottin and comprises a donor scaffold and a donor interaction sequence, and

the recipient diversity scaffold domain is an antibody variable domain and comprises a recipient scaffold and a recipient interaction sequence, and

wherein one, two or all three of the donor interaction sequence, the recipient interaction sequence and the linkers are diverse in said founder library,

(ii) screening the founder library for binding members which display a binding activity, and

(iii) identifying one or more binding members in the founder library which display the binding activity.

An example of the disclosure provides a method of screening comprising;

(i) providing a founder library of binding members,

each binding member in the founder library comprising a fusion protein and optionally, a partner domain associated with the fusion protein,

wherein the fusion protein comprises a donor diversity scaffold domain inserted within a recipient diversity scaffold domain, optionally wherein the N and C terminals of the donor diversity scaffold domain are each linked to the recipient diversity scaffold domain with linkers of 4 amino acids or fewer each,

wherein the donor diversity scaffold domain comprises a donor scaffold and a donor interaction sequence and the recipient diversity scaffold domain comprises a recipient scaffold and a recipient interaction sequence, and one or more of the donor interaction sequence, recipient interaction sequence and the linkers are diverse in said founder library,

(ii) screening the founder library for founder binding members which display binding activity, and

(iii) identifying one or more founder binding members in the founder library which display the binding activity.

In some examples, the linkers are diverse in said founder library.

The method may further comprise;

(iv) introducing diverse amino acid residues at one or more positions in the amino acid sequence of one or more identified founder binding members to produce a modified library of binding members,

(v) screening the modified library for modified binding members which display a binding activity and

(vi) identifying one or more modified binding members in the modified library which display the binding activity.

In some examples, diverse amino acid residues may be introduced into the donor interaction sequence, recipient interaction sequence and/or partner domain of the one or more identified founder binding members.

The method may further comprise;

(vii) associating the fusion protein from the one or more identified binding members or modified binding members with a diverse population of partner binding domains (partner chains) to produce a shuffled library of binding members,

(viii) screening the shuffled library for shuffled binding members which display a binding activity,

(ix) identifying one or more shuffled binding members which display the binding activity.

In another aspect, the present disclosure refers to a method of producing a library of binding members comprising;

providing a population of nucleic acids encoding a diverse population of fusion proteins comprising a donor diversity scaffold domain inserted into a recipient diversity scaffold domain,

wherein the donor diversity scaffold domain is a cysteine-rich peptide consisting of 100 or fewer amino acids, comprising two or more disulphide bridges formed by four or more cysteine residues within said donor diversity scaffold domain, wherein the cysteine-rich peptide is an ion channel modulating peptide, a venom peptide or a knottin and comprises a donor scaffold and a donor interaction sequence, and

the recipient diversity scaffold domain is an antibody variable domain and comprises a recipient scaffold and a recipient interaction sequence, optionally wherein the N and C terminals of the donor diversity scaffold domain are linked to the recipient diversity scaffold domain with linkers of up to 4 amino acids, and

wherein one, two or all three of the donor interaction sequence, the recipient interaction sequence and the linkers are diverse in said population,

expressing said population of nucleic acids to produce the diverse population, and

optionally associating the fusion proteins with a population of partner domains,

thereby producing a library of binding members.

Another example of the disclosure provides a method of producing a library of binding members comprising;

providing a population of nucleic acids encoding a diverse population of fusion proteins comprising a donor diversity scaffold domain inserted into a recipient diversity

scaffold domain, optionally wherein the N and C terminals of the donor diversity scaffold domain are linked to the recipient diversity scaffold domain with linkers of 4 amino acids or fewer at each terminal,

wherein the donor diversity scaffold domain comprises a donor scaffold and a donor interaction sequence and the recipient diversity scaffold domain comprises a recipient scaffold and a recipient interaction sequence, and

one or more of the donor interaction sequence, recipient interaction sequence and linkers are diverse in said population,

expressing said population of nucleic acids to produce the diverse population, and

optionally associating the fusion proteins with a population of partner domains.

thereby producing a library of binding members.

In yet another aspect, the present disclosure refers to a library of binding members comprising;

a diverse population of fusion proteins comprising a donor diversity scaffold domain inserted into a recipient diversity scaffold domain,

wherein the donor diversity scaffold domain is a cysteine-rich peptide consisting of 100 or fewer amino acids, comprising two or more disulphide bridges formed by four or more cysteine residues within said donor diversity scaffold domain, wherein the cysteine-rich peptide is an ion channel modulating peptide, a venom peptide or a knottin and comprises a donor scaffold and a donor interaction sequence, and

the recipient diversity scaffold domain is an antibody variable domain and comprises a recipient scaffold and a recipient interaction sequence, optionally wherein the N and C terminals of the donor diversity scaffold domain are linked to the recipient diversity scaffold domain with linkers of up to 4 amino acids, and

one, two or all three the donor interaction sequence, the recipient interaction sequence and the linkers are diverse in said population, and

optionally wherein the fusion proteins are associated with binding partners to form heterodimers.

Another example of the disclosure provides a library of binding members comprising;

a diverse population of fusion proteins comprising a donor diversity scaffold domain inserted into a recipient diversity scaffold domain,

wherein the donor diversity scaffold domain comprises a donor scaffold and a donor interaction sequence and the recipient diversity scaffold domain comprises a recipient scaffold and a recipient interaction sequence, optionally wherein the N and C terminals of

the donor diversity scaffold domain are each linked to the recipient diversity scaffold domain with linkers of 4 amino acids or fewer,

wherein the donor diversity scaffold domain comprises a donor scaffold and a donor interaction sequence and the recipient diversity scaffold domain comprises a recipient scaffold and a recipient interaction sequence, and

one or more of the donor interaction sequence, recipient interaction sequence and linkers are diverse in said population, and

optionally wherein the fusion proteins are associated with binding partners to form heterodimers.

In yet another aspect, the present disclosure refers to a fusion protein comprising a donor diversity scaffold domain inserted into a recipient diversity scaffold domain wherein

the donor diversity scaffold domain is a cysteine-rich peptide consisting of 100 or fewer amino acids, comprising two or more disulphide bridges formed by four or more cysteine residues within said donor diversity scaffold domain, wherein the cysteine-rich peptide is an ion channel modulating peptide, a venom peptide or a knottin and comprises a donor scaffold and a donor interaction sequence and

the recipient diversity scaffold domain is an antibody variable domain and comprises a recipient scaffold and a recipient interaction sequence.

Another example of the disclosure provides a fusion protein comprising a donor diversity scaffold domain inserted into a recipient diversity scaffold domain

wherein the donor diversity scaffold domain comprises a donor scaffold and a donor interaction sequence and the recipient diversity scaffold domain comprises a recipient scaffold and a recipient interaction sequence, optionally wherein the N and C terminals of the donor diversity scaffold domain are linked to the recipient diversity scaffold domain with linkers of 4 amino acids or fewer.

Another aspect of the disclosure provides a binding member comprising a fusion protein described herein and a partner domain associated with the fusion protein.

In yet another aspect, the present disclosure refers to a method of producing a binding member comprising;

inserting a nucleic acid encoding a donor diversity scaffold domain into a nucleic acid encoding a recipient diversity scaffold domain to produce a chimeric nucleic acid encoding a fusion protein;

wherein the donor diversity scaffold domain is a cysteine-rich peptide consisting of 100 or fewer amino acids, comprising two or more disulphide bridges formed by four or more cysteine residues within said donor diversity scaffold domain, wherein the cysteine-rich peptide is an ion channel modulating peptide, a venom peptide or a knottin and comprises a donor scaffold and a donor interaction sequence, and

the recipient diversity scaffold domain is an antibody variable domain and comprises a recipient scaffold and a recipient interaction sequence;

expressing said chimeric nucleic acid to produce the fusion protein, and

optionally, associating the fusion protein with a partner domain.

In yet another aspect, the present disclosure refers to a nucleic acid encoding a fusion protein as described herein or a binding member as described herein. In still another aspect, the present disclosure refers to a population of nucleic acids encoding a library as described herein. In still another aspect, the present disclosure refers to a population of viral particles or host cells comprising a library as described herein.

Other examples of the disclosure provide isolated donor diversity scaffold domains, recipient diversity scaffold domains or partner domains from binding members identified and/or isolated as described herein, for example isolated donor diversity scaffold domains engineered as described herein or isolated recipient diversity scaffold domains or partner domains which may be useful in heterologous binding members, for example binding members which do not include the original donor diversity scaffold domain.

Brief Description of Figures

Figure 1A shows a representation of pIONTAS1, showing the cassette encompassing the lacI promoter, M13 leader sequence scFv antibody gene-gene 3 (not to scale). This cassette is present within the Hind3/EcoR1 cloning sites of pUC119 which has an Ampicillin resistance gene and a coleE1 origin and an f1 bacteriophage origin of replication. The VH gene is flanked by an Nco1 and Xho1 restriction site and the VL gene is flanked by Nhe1 and Not 1 restriction sites allowing simple cloning into compatible vectors. Figure 1B shows a representation of the VL domain showing positions of framework regions (FW) and CDR regions. A knottin donor has been inserted at CDR1 position of the VL recipient. Pml1 and Mfe1 sites allow cloning of the knottin donor library. The region from FR2 to the end of FR4 is derived from a repertoire of light chains[28]. Figure 1C is as described for Figure 1B but with the knottin donor replacing CDR2 of the VL gene using Pst1 and BspE1 sites.

Figure 2 shows the sequence of synthetic single chain Fv genes used in KnotBody construction. The VH of an existing antibody D12 was used within KnotBody constructs. Figure 2A shows restriction sites Nco1 and Xho1 which flank the VH sequence as well as the flexible linker joining VH to VL. This is followed by a sequence encoding the Vlamda1a (IGLV1-36) germline. CDR1 of the VL is shown and is preceded by a Pml1 cloning site. A Ser to Thr mutation was introduced to enable the inclusion of a Pml1 site at the end of framework 1. This site allows cloning of the knottin into the CDR1 position of the VL. At the end of VL framework 2 of the synthetic gene is a Pst1 site allowing cloning of a knottin into the CDR2 position. Figure 2B is as for Figure 2A but showing the V kappa germline sequence IGKV1D-39

Figure 3 shows the insertion of EETI-II donor knottin into VL recipient. Figure 3A shows the sequence of knottin donor EETI-II with cysteine residues emboldened and core sequence involved in trypsin binding (“PRIL”) underlined. Figure 3B shows a representation of the insertion of EETI-II donor into CDR1 or CDR2 of the lambda germline gene IGLV1-36. Boundaries are defined according to the international ImMunoGeneTics information system (IMGT)[29]. Amino acids are represented by IUPAC single letter amino acids code. Figure 3Bi

Claims

1. A method of screening comprising;

(i) providing a founder library of binding members,

each binding member in the library comprising a fusion protein and optionally, a partner domain associated with the fusion protein,

wherein the fusion protein comprises a donor diversity scaffold domain inserted within a recipient diversity scaffold domain, optionally wherein the N and C terminals of the donor diversity scaffold domain are linked to the recipient diversity scaffold domain with linkers of up to 4 amino acids,

wherein the donor diversity scaffold domain is a cysteine-rich peptide consisting of 100 or fewer amino acids, comprising two or more disulphide bridges formed by four or more cysteine residues within said donor diversity scaffold domain, wherein the cysteine-rich peptide is an ion channel modulating peptide, a venom peptide or a knottin and comprises a donor scaffold and a donor interaction sequence, and

the recipient diversity scaffold domain is an antibody variable domain and comprises a recipient scaffold and a recipient interaction sequence, and

wherein one, two or all three of the donor interaction sequence, the recipient interaction sequence and the linkers are diverse in said founder library,

(ii) screening the founder library for binding members which display a binding activity, and

(iii) identifying one or more binding members in the founder library which display the binding activity.

2. A method according to claim 1 comprising further enriching the one or more binding members by;

(a) recovering the one or more binding members,

(b) subjecting the recovered binding members to selection for the binding activity,

(c) recovering the one or more selected binding members

(d) optionally repeating steps (b) and (c) one or more times.

3. A method according to claim 1 or claim 2 further comprising;

(iv) introducing diverse amino acid residues at one or more positions in the amino acid sequence of one or more identified founder binding members to produce a modified library of binding members,

(v) screening the modified library for modified binding members which display a binding activity and

(vi) identifying one or more modified binding members in the modified library which display the binding activity.

4. A method according to claim 3 comprising further enriching the one or more binding members by;

(e) recovering the one or more modified binding members,

(f) subjecting the recovered modified binding members to selection for the binding activity,

(g) recovering the one or more selected binding modified members and

(h) optionally repeating steps (f) and (g) one or more times.

5. A method according to any one of the preceding claims comprising;

(vii) associating the fusion protein from the one or more identified binding members or modified binding members with a diverse population of binding partners to produce a shuffled library of binding members,

(viii) screening the shuffled library for shuffled binding members which display a binding activity,

(ix) identifying one or more shuffled binding members which display the binding activity;

and optionally further enriching the one or more shuffled binding members by;

(i) recovering the one or more shuffled binding members,

(j) subjecting the recovered shuffled binding members to selection for the binding activity,

(k) recovering the one or more selected binding modified members and

(l) optionally repeating steps (j) and (k) one or more times.

6. A method according to any one of the preceding claims comprising introducing an additional amino acid sequence into the fusion proteins or partner domains of the one or more identified binding members from the founder library, modified library and/or shuffled library.

7. A method according to any one of the preceding claims wherein the binding activity is binding to a target molecule, wherein one of the donor diversity scaffold domain, recipient diversity scaffold domain and partner domain binds to the target molecule and the method

comprises modifying or replacing said donor diversity scaffold domain, recipient diversity scaffold domain or partner domain in the one or more identified binding members.

8. A method according to any one of the preceding claims wherein the method comprises isolating and/or purifying the one or more identified binding members from the library, the modified library and/or the shuffled library.

9. A method according to any one of the preceding claims wherein the binding members in the library are displayed on the surface of a ribosome, cell or virus which comprises the nucleic acid encoding the binding member.

10. A method according to claim 9 wherein the method comprises isolating and/or purifying the ribosome, cell or virus displaying the one or more identified binding members from the library, the modified library and/or the shuffled library.

11. A method according to claim 9 or 10 wherein the method comprises isolating and/or purifying the nucleic acid encoding the one or more identified binding members from the library, the modified library and/or the shuffled library.

12. A method according to any one of claims 9 to 11 wherein the method comprises amplifying and/or cloning the nucleic acid encoding the one or more identified binding members from the library, the modified library and/or the shuffled library, and optionally sequencing the nucleic acid encoding the one or more identified binding members from the library, the modified library and/or the shuffled library.

13. A method of producing a library of binding members comprising;

providing a population of nucleic acids encoding a diverse population of fusion proteins comprising a donor diversity scaffold domain inserted into a recipient diversity scaffold domain,

wherein the donor diversity scaffold domain is a cysteine-rich peptide consisting of 100 or fewer amino acids, comprising two or more disulphide bridges formed by four or more cysteine residues within said donor diversity scaffold domain, wherein the cysteine-rich peptide is an ion channel modulating peptide, a venom peptide or a knottin and comprises a donor scaffold and a donor interaction sequence, and

the recipient diversity scaffold domain is an antibody variable domain and comprises a recipient scaffold and a recipient interaction sequence, optionally wherein the N and C

terminals of the donor diversity scaffold domain are linked to the recipient diversity scaffold domain with linkers of up to 4 amino acids, and

wherein one, two or all three of the donor interaction sequence, the recipient interaction sequence and the linkers are diverse in said population,

expressing said population of nucleic acids to produce the diverse population, and

optionally associating the fusion proteins with a population of partner domains,

thereby producing a library of binding members.

14. A library of binding members comprising;

a diverse population of fusion proteins comprising a donor diversity scaffold domain inserted into a recipient diversity scaffold domain,

wherein the donor diversity scaffold domain is a cysteine-rich peptide consisting of 100 or fewer amino acids, comprising two or more disulphide bridges formed by four or more cysteine residues within said donor diversity scaffold domain, wherein the cysteine-rich peptide is an ion channel modulating peptide, a venom peptide or a knottin and comprises a donor scaffold and a donor interaction sequence, and

the recipient diversity scaffold domain is an antibody variable domain and comprises a recipient scaffold and a recipient interaction sequence, optionally wherein the N and C terminals of the donor diversity scaffold domain are linked to the recipient diversity scaffold domain with linkers of up to 4 amino acids, and

one, two or all three the donor interaction sequence, the recipient interaction sequence and the linkers are diverse in said population, and

optionally wherein the fusion proteins are associated with binding partners to form heterodimers.

15. A fusion protein comprising a donor diversity scaffold domain inserted into a recipient diversity scaffold domain wherein

the donor diversity scaffold domain is a cysteine-rich peptide consisting of 100 or fewer amino acids, comprising two or more disulphide bridges formed by four or more cysteine residues within said donor diversity scaffold domain, wherein the cysteine-rich peptide is an ion channel modulating peptide, a venom peptide or a knottin and comprises a donor scaffold and a donor interaction sequence and

the recipient diversity scaffold domain is an antibody variable domain and comprises a recipient scaffold and a recipient interaction sequence.

16. A binding member comprising a fusion protein according to claim 15 and a partner domain.

17. A method of producing a binding member comprising;

inserting a nucleic acid encoding a donor diversity scaffold domain into a nucleic acid encoding a recipient diversity scaffold domain to produce a chimeric nucleic acid encoding a fusion protein;

wherein the donor diversity scaffold domain is a cysteine-rich peptide consisting of 100 or fewer amino acids, comprising two or more disulphide bridges formed by four or more cysteine residues within said donor diversity scaffold domain, wherein the cysteine-rich peptide is an ion channel modulating peptide, a venom peptide or a knottin and comprises a donor scaffold and a donor interaction sequence, and

the recipient diversity scaffold domain is an antibody variable domain and comprises a recipient scaffold and a recipient interaction sequence;

expressing said chimeric nucleic acid to produce the fusion protein, and

optionally, associating the fusion protein with a partner domain.

18. A method, library, fusion protein or binding member according to any of the preceding claims wherein the recipient diversity scaffold domain is an antibody light chain variable (VL) domain.

19. A method, library, fusion protein or binding member according to claim 18 wherein the partner domain is an antibody heavy chain variable (VH) domain.

20. A method, library, fusion protein or binding member according to any of claims 1 to 19 wherein the recipient diversity scaffold domain is an antibody heavy chain variable (VH) domain.

21. A method, library, fusion protein or binding member according to claim 20 wherein the partner domain is an antibody light chain variable (VL) domain.

22. A method, library, fusion protein or binding member according to any one of the preceding claims wherein therein the donor diversity scaffold domain replaces all or part of VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 or VL CDR3 of the antibody variable domain.

23. A method, library, fusion protein or binding member according to claim 21 wherein the donor diversity scaffold domain replaces all or part of the CDR1 or CDR2 of the antibody VL domain.

24. A method, library, fusion protein or binding member according to any one of the preceding claims wherein the donor diversity scaffold domain consists of at least 15 amino acids, preferably at least 20 amino acids.

25. A method, library, fusion protein or binding member according to any one of the preceding claims, wherein the donor diversity scaffold domain is a venom peptide.

26. A method, library, fusion protein or binding member according to any one of the preceding claims, wherein the donor diversity scaffold domain is a knottin.

27. A method, library, fusion protein or binding member according to any one of the preceding claims wherein the donor diversity scaffold domain is Ecballium elaterium trypsin inhibitor-II (EETI-II), MCoTI-II, Huwentoxin-IV, ProTx-II, SSm6a, Kaliotoxin, MoKa-1, Shk, PcTx1, Conotoxin-ω, or a variant thereof.

28. A method, library, fusion protein or binding member according to any one of the preceding claims wherein the native N and C terminals of the donor diversity scaffold domain are linked to the recipient diversity scaffold domain.

29. A method, library, fusion protein or binding member according to any one of claims 1 to 27 wherein artificial N and C terminals generated by cyclisation and linearization of the donor diversity scaffold domain are linked to the recipient diversity scaffold domain.

30. A nucleic acid encoding a fusion protein according to any one of claims 15 and 18 to 29 or a binding member according to any one of claims 20 and 22 to 29.

31. A population of nucleic acids encoding a library according to any one of claims 14 and 17 to 29.

32. A population of viral particles or host cells comprising a library according to any one of claims 22 and 17 to 29 or a population according to claim 31.