this fashion is the spread of toxin away from the administration site and into surrounding tissue or systemic circulation. The diffusion of toxin away from the target tissue is believed to be responsible for undesirable side effects that in extreme cases may be life threatening. This can be a particular concern when using clostridial toxin therapeutics (such as BoNT therapeutics) at high doses, concentrations and injection volumes. Adverse effects associated with this problem that have been reported for commercial BoNT/A therapeutics include asthenia, generalised muscle weakness, diplopia, ptosis, dysphagia, dysphonia, dysarthria, urinary incontinence, and breathing difficulties. Swallowing and breathing difficulties can be life threatening and there have been reported deaths related to the spread of toxin effects.

There is therefore a need in the art for clostridial toxins which have properties of increased tissue retention at the site of administration, and which accordingly exhibit a reduction in diffusion away from the administration site, as compared to known clostridial toxins.

Summary

The present disclosure solves the above problem by providing engineered clostridial toxins, as specified in the claims.

In one aspect, the present disclosure refers to a di-chain engineered botulinum neurotoxin serotype A (BoNT/A) comprising a light chain and heavy chain joined together by a disulphide bond,

wherein the di-chain engineered BoNT/A retains the abilities of target cell binding, translocation, and cleavage of a target SNARE protein,

wherein the heavy chain comprises a clostridial toxin receptor binding domain (HC domain) that has been modified from the HC domain of BoNT/A shown as SEQ ID NO: 2 by at least four amino acid substitutions located in said HC domain,

wherein the at least four amino acid substitutions located in said HC domain increase the isoelectric point (pI) of the di-chain engineered BoNT/A to a value that is at least 0.2 pI units higher than the pI of an otherwise identical di-chain BoNT/A lacking the at least four amino acid substitutions located in said HC domain, and

wherein the at least four amino acid substitutions located in said HC domain comprise substitutions at four or more amino acids selected from: ASN 886, ASN 905, GLN 915, ASN 918, GLU 920, ASN 930, ASN 954, SER 955, GLN 991, GLU 992, GLN 995, ASN 1006, ASN 1025, ASN 1026, ASN 1032, ASN 1043, ASN 1046, ASN 1052, ASP 1058, HIS 1064, ASN 1080, GLU 1081, GLU 1083, ASP 1086, ASN 1188, ASP 1213, GLY 1215, ASN 1216, GLN 1229, ASN 1242, ASN 1243, SER 1274, and THR 1277 with a lysine residue or an arginine residue.

In yet another aspect, the present disclosure refers to a single-chain engineered botulinum neurotoxin serotype A (BoNT/A) comprising a light chain and heavy chain,

wherein the heavy chain comprises a clostridial toxin receptor binding domain (HC domain) that has been modified from the HC domain of BoNT/A shown as SEQ ID NO: 2 by at least four amino acid substitutions located in said HC domain,

wherein the at least four amino acid substitutions located in said HC domain increase the isoelectric point (pI) of the single-chain engineered BoNT/A to a value that is at least 0.2 pI units higher than the pI of an otherwise identical single-chain BoNT/A lacking the at least four amino acid substitutions located in said HC domain, and

wherein the at least four amino acid substitutions located in said HC domain comprise substitutions at four or more amino acids selected from: ASN 886, ASN 905, GLN 915, ASN 918, GLU 920, ASN 930, ASN 954, SER 955, GLN 991, GLU 992, GLN 995, ASN 1006, ASN 1025, ASN 1026, ASN 1032, ASN 1043, ASN 1046, ASN 1052, ASP 1058, HIS 1064, ASN 1080, GLU 1081, GLU 1083, ASP 1086, ASN 1188, ASP 1213, GLY 1215, ASN 1216, GLN 1229, ASN 1242, ASN 1243, SER 1274, and THR 1277 with a lysine residue or an arginine residue.

In yet another aspect, the present disclosure refers to a nucleic acid comprising a nucleic acid sequence encoding an engineered clostridial toxin as disclosed herein.

In yet another aspect, the present disclosure refers to an *in vitro* method of producing a single-chain engineered clostridial toxin having a light chain and a heavy chain, the method comprising expressing a nucleic acid as disclosed herein in a suitable host cell, lysing the host cell to provide a host cell homogenate containing the single-chain engineered clostridial toxin, and isolating the single-chain engineered clostridial toxin

In yet another aspect, the present disclosure refers to a method of activating an engineered clostridial toxin, the method comprising providing a single-chain engineered clostridial toxin obtainable by the method as disclosed herein, contacting the single-chain engineered clostridial toxin with a protease that cleaves the single-chain engineered clostridial toxin at a recognition site (cleavage site) located between the light chain and heavy chain, and converting the single-chain engineered clostridial toxin into a di-chain engineered clostridial toxin wherein the light chain and heavy chain are joined together by a disulphide bond.

In yet another aspect, the present disclosure refers to a di-chain engineered clostridial toxin obtainable by the method as disclosed herein.

In yet another aspect, the present disclosure refers to a di-chain engineered clostridial toxin as disclosed herein, for use in medicine.

In yet another aspect, the present disclosure refers to a method of cosmetic treatment benefitting from cell/muscle incapacitation comprising that a di-chain engineered clostridial toxin as disclosed herein is to be administered.

Detailed Description

In one aspect, the invention provides an engineered clostridial toxin comprising at least one amino acid modification, wherein said at least one amino acid modification increases the isoelectric point (pI) of the engineered clostridial toxin to a value that is at least 0.2 (for example, at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical clostridial toxin lacking said at least one amino acid modification. In one embodiment, said at least one amino acid modification increases the pI of the engineered clostridial toxin to a value that is at least 0.4 pI units higher than the pI of an otherwise identical clostridial toxin lacking said at least one amino acid modification. In one embodiment, said at least one amino acid modification increases the pI of the engineered clostridial toxin to a value that is at least 0.5 pI units higher than the pI of an otherwise identical clostridial toxin lacking said at least one amino acid modification. In one embodiment, said at least one amino acid modification increases the pi of the engineered clostridial toxin to a value that is at least 0.6 pI units higher than the pi of an otherwise identical clostridial toxin lacking said at least one amino acid modification. In one embodiment, said at

Claims

1. A di-chain engineered botulinum neurotoxin serotype A (BoNT/A) comprising a light chain and heavy chain joined together by a disulphide bond,

wherein the di-chain engineered BoNT/A retains the abilities of target cell binding, translocation, and cleavage of a target SNARE protein,

wherein the heavy chain comprises a clostridial toxin receptor binding domain (H_C domain) that has been modified from the H_C domain of BoNT/A shown as SEQ ID NO: 2 by at least four amino acid substitutions located in said H_C domain,

wherein the at least four amino acid substitutions located in said H_C domain increase the isoelectric point (pI) of the di-chain engineered BoNT/A to a value that is at least 0.2 pI units higher than the pI of an otherwise identical di-chain BoNT/A lacking the at least four amino acid substitutions located in said H_C domain, and

wherein the at least four amino acid substitutions located in said H_C domain comprise substitutions at four or more amino acids selected from: ASN 886, ASN 905, GLN 915, ASN 918, GLU 920, ASN 930, ASN 954, SER 955, GLN 991, GLU 992, GLN 995, ASN 1006, ASN 1025, ASN 1026, ASN 1032, ASN 1043, ASN 1046, ASN 1052, ASP 1058, HIS 1064, ASN 1080, GLU 1081, GLU 1083, ASP 1086, ASN 1188, ASP 1213, GLY 1215, ASN 1216, GLN 1229, ASN 1242, ASN 1243, SER 1274, and THR 1277 with a lysine residue or an arginine residue.

2. A single-chain engineered botulinum neurotoxin serotype A (BoNT/A) comprising a light chain and heavy chain,

wherein the heavy chain comprises a clostridial toxin receptor binding domain (H_C domain) that has been modified from the H_C domain of BoNT/A shown as SEQ ID NO: 2 by at least four amino acid substitutions located in said H_C domain,

wherein the at least four amino acid substitutions located in said H_C domain increase the isoelectric point (pI) of the single-chain engineered BoNT/A to a value that is at least 0.2 pI units higher than the pI of an otherwise identical single-chain BoNT/A lacking the at least four amino acid substitutions located in said H_C domain, and

wherein the at least four amino acid substitutions located in said H_C domain comprise substitutions at four or more amino acids selected from: ASN 886, ASN 905, GLN 915, ASN 918, GLU 920, ASN 930, ASN 954, SER 955, GLN 991, GLU 992, GLN 995, ASN 1006, ASN 1025, ASN 1026, ASN 1032, ASN 1043, ASN 1046, ASN 1052, ASP 1058, HIS 1064,

ASN 1080, GLU 1081, GLU 1083, ASP 1086, ASN 1188, ASP 1213, GLY 1215, ASN 1216, GLN 1229, ASN 1242, ASN 1243, SER 1274, and THR 1277 with a lysine residue or an arginine residue.

3. A nucleic acid comprising a nucleic acid sequence encoding an engineered clostridial toxin according to claim 2.

4. An *in vitro* method of producing a single-chain engineered clostridial toxin having a light chain and a heavy chain, the method comprising expressing a nucleic acid according to claim 3 in a suitable host cell, lysing the host cell to provide a host cell homogenate containing the single-chain engineered clostridial toxin, and isolating the single-chain engineered clostridial toxin.

5. A method of activating an engineered clostridial toxin, the method comprising providing a single-chain engineered clostridial toxin obtainable by the method of claim 4, contacting the single-chain engineered clostridial toxin with a protease that cleaves the single-chain engineered clostridial toxin at a recognition site (cleavage site) located between the light chain and heavy chain, and converting the single-chain engineered clostridial toxin into a di-chain engineered clostridial toxin wherein the light chain and heavy chain are joined together by a disulphide bond.

6. A di-chain engineered clostridial toxin obtainable by the method of claim 5.

7. A di-chain engineered clostridial toxin according to claim 1 or 6, for use in medicine.

8. A method of cosmetic treatment benefitting from cell/muscle incapacitation comprising that a di-chain engineered clostridial toxin according to claim 1 or 6 is to be administered.

9. The method according to claim 8, wherein the cosmetic treatment comprises treating brow furrows and/or skin wrinkles.