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 an engineered clostridial toxin comprising between 4 and 40 amino acid modifications located in the clostridial toxin H_{CN} domain, wherein said between 4 and 40 amino acid modifications increase the isoelectric point (pI) of the engineered clostridial toxin to a value that is at least 0.2 pI units higher than the pI of an otherwise identical clostridial toxin lacking said between 4 and 40 amino acid modifications, wherein the engineered clostridial toxin is:

a. an engineered botulinum neurotoxin A (BoNT/A) having a pI of at least 6.6 and the between 4 and 40 amino acid modifications comprise modification of at least 4 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, and ASP 1086;

b. an engineered BoNT/B having a pI of at least 5.5 and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 873, ASN 874, GLU 892, ASP 895, ASN 906, ASP 940, ASN 948, GLU 949, ASN 958, ASN 959, ASN 979, ASN 990, GLU 993, ASP 994, GLU 997, ASN 1012, ASN 1019, ASP 1030, ASP 1047, ASP 1049, GLU 1065, and GLU 1072;

c. an engineered BoNT/C_1 having a pI of at least 5.7 and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 881, ASP 898, GLU 916, GLU 927, ASN 952, ASN 964, ASN 965, ASN 984, GLU 985, ASP 986, ASP 996, ASN 1000, GLU 1036, ASN 1041, ASP 1062, ASP 1064, GLU 1079, and ASP 1081;

d. an engineered BoNT/D having a pI of at least 5.7 and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 877, ASP 893, ASN 894, ASN 898, ASN 920, ASN 945, ASN 948, GLU 957, GLN 958, ASN 959, ASN 968, ASN 979, GLU 1030, ASP 1031, ASP 1033, GLU 1047, GLU 1051, ASN 1052, and GLU 1066;

e. an engineered BoNT/E having a pI of at least 6.2 and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 859, ASP 860, ASN 892, ASP 893, ASP 904, ASP 909, ASN 928, ASN 932, ASN 934, ASN 935, GLU 936, ASP 945, ASN 946, ASN 947, ASN 966, ASN 976, ASN 979, ASN 981, ASP 985, GLN 1014, ASN 1019, ASN 1022, ASP 1027, and ASN 1035;

f. an engineered BoNT/F having a pI of at least 5.8 and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 879, ASP 896, ASN 922, ASN 923, ASN 928, ASN 947, ASN 950, ASN 952, ASN 953, GLU 954, ASN 963, ASN 964, ASN 965, ASN 987, GLN 997, ASN 1037, ASP 1040, ASP 1045, ASN 1055, and ASP 1056;

g. an engineered BoNT/G having a pI of at least 5.4 and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASP 900, ASN 909, ASN 910, GLU 912, ASN 913, ASN 945, ASN 947, GLU 956, ASN 965, ASP 966, ASN 986, ASN 1001, ASN 1038, ASP 1040, ASN 1046, ASP 1057, GLU 1073, ASN 1075, and ASN 1090; or

h. an engineered tetanus neurotoxin (TeNT) having a pI of at least 6.0 and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 893, ASP 894, ASP 911, ASN 919, ASN 927, ASN 928, GLU 929, GLN 968, ASN 972, GLU 973, GLU 1010, ASP 1018, ASN 1079, ASN 1080, ASN 1081, and ASN 1097; and

wherein the between 4 and 40 amino acid modifications are selected from:

i. substitution of an acidic amino acid residue with a basic amino acid residue;
ii. substitution of an acidic amino acid residue with an uncharged amino acid residue;
iii. substitution of an uncharged amino acid residue with a basic amino acid residue;
iv. insertion of a basic amino acid residue; or
v. deletion of an acidic amino acid residue; and

wherein the engineered clostridial toxin has a Safety Ratio of at least 8, wherein the Safety Ratio is calculated as: dose of toxin required for -10% bodyweight change measured as pg/mouse divided by DAS ED_{50} measured as pg/mouse, wherein ED_{50} = dose required to produce a DAS score of 2.

In another aspect, the present disclosure refers to a method for producing an engineered clostridial toxin, the method comprising modifying an H_{CN} domain of a clostridial toxin to introduce between 4 and 40 amino acid modifications, thereby producing the engineered clostridial toxin,

wherein the between 4 and 40 amino acid modifications increase the isoelectric point (pI) of the engineered clostridial toxin to a value that is at least 0.2 pI units higher than the pI of an otherwise identical clostridial toxin lacking the between 4 and 40 amino acid modifications, and

wherein the clostridial toxin is a botulinum neurotoxin A (BoNT/A) and the between 4 and 40 amino acid modifications comprise modification of at least 4 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 1026, ASN 1032, ASN 1043, ASN 1046, ASN 1052, ASP 1058, HIS 1064, ASN 1080, GLU 1081, GLU 1083, and ASP 1086, or

wherein the clostridial toxin is a BoNT/B and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 873, ASN 874, GLU 892, ASP 895, ASN 906, ASP 940, ASN 948, GLU 949, ASN 958, ASN 959, ASN 979, ASN 990, GLU 993, ASP 994, GLU 997, ASN 1012, ASN 1019, ASP 1030, ASP 1047, ASP 1049, GLU 1065, and GLU 1072, or

wherein the clostridial toxin is a BoNT/C_1 and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 881, ASP 898, GLU 916, GLU 927, ASN 952, ASN 964, ASN 965, ASN 984, GLU 985, ASP 986, ASP 996, ASN 1000, GLU 1036, ASN 1041, ASP 1062, ASP 1064, GLU 1079, and ASP 1081, or

wherein the clostridial toxin is a BoNT/D and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 877, ASP 893, ASN 894, ASN 898, ASN 920, ASN 945, ASN 948, GLU 957, GLN 958, ASN 959, ASN 968, ASN 979, GLU 1030, ASP 1031, ASP 1033, GLU 1047, GLU 1051, ASN 1052, and GLU 1066, or

wherein the clostridial toxin is a BoNT/E and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 859, ASP 860, ASN 892, ASP 893, ASP 904, ASP 909, ASN 928, ASN 932, ASN 934, ASN 935, GLU 936, ASP 945, ASN 946, ASN 947, ASN 966, ASN 976, ASN 979, ASN 981, ASP 985, GLN 1014, ASN 1019, ASN 1022, ASP 1027, and ASN 1035, or

wherein the clostridial toxin is a BoNT/F and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 879, ASP 896, ASN 922, ASN 923, ASN 928, ASN 947, ASN 950, ASN 952, ASN 953, GLU 954, ASN 963, ASN 964, ASN 965, ASN 987, GLN 997, ASN 1037, ASP 1040, ASP 1045, ASN 1055, and ASP 1056, or

wherein the clostridial toxin is a BoNT/G and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASP 900, ASN 909, ASN 910, GLU 912, ASN 913, ASN 945, ASN 947, GLU 956, ASN 965, ASP 966, ASN 986, ASN 1001, ASN 1038, ASP 1040, ASN 1046, ASP 1057, GLU 1073, ASN 1075, and ASN 1090, or

wherein the clostridial toxin is a tetanus neurotoxin (TeNT) and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 893, ASP 894, ASP 911, ASN 919, ASN 927, ASN 928, GLU 929, GLN 968, ASN 972, GLU 973, GLU 1010, ASP 1018, ASN 1079, ASN 1080, ASN 1081, and ASN 1097, and

wherein the between 4 and 40 amino acid modifications are selected from:

i. substitution of an acidic amino acid residue with a basic amino acid residue;
ii. substitution of an acidic amino acid residue with an uncharged amino acid residue;
iii. substitution of an uncharged amino acid residue with a basic amino acid residue;
iv. insertion of a basic amino acid residue; and
v. deletion of an acidic amino acid residue.

wherein the engineered clostridial toxin has a Safety Ratio of at least 8, wherein the Safety Ratio is calculated as: dose of toxin required for -10% bodyweight change measured as pg/mouse divided by DAS ED_{50} measured as pg/mouse, wherein ED_{50} = dose required to produce a DAS score of 2.

In another aspect, the present disclosure refers to a method for producing an engineered clostridial toxin, the method comprising modifying a clostridial toxin receptor binding domain (H_C domain) of a clostridial toxin to introduce at least four amino acid modifications, thereby producing the engineered clostridial toxin,

wherein the at least four amino acid modifications increase the isoelectric point (pI) of the engineered clostridial toxin to a value that is at least 0.2 pI units higher than the pI of the otherwise identical clostridial toxin lacking the at least four amino acid modifications, and

wherein the clostridial toxin is botulinum neurotoxin A (BoNT/A) and the at least four amino acid modifications consist of modification of at least four 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, or

wherein the clostridial toxin is BoNT/B and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 873, ASN 874, GLU 892, ASP 895, ASN 906, ASP 940, ASN 948, GLU 949, ASN 958, ASN 959, ASN 979, ASN 990, GLU 993, ASP 994, GLU 997, ASN 1012, ASN 1019, ASP 1030, ASP 1047, ASP 1049, GLU 1065, GLU 1072, GLN 1176, GLU 1189, GLU 1252, and ASN 1273, or

wherein the clostridial toxin is BoNT/C_1 and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 881, ASP 898, GLU 916, GLU 927, ASN 952, ASN 964, ASN 965, ASN 984, GLU 985, ASP 986, ASP 996, ASN 1000, GLU 1036, ASN 1041, ASP 1062, ASP 1064, GLU 1079, and ASP 1081, or

wherein the clostridial toxin is BoNT/D and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 877, ASP 893, ASN 894, ASN 898, ASN 920, ASN 945, ASN 948, GLU 957, GLN 958, ASN 959, ASN 968, ASN 979, GLU 1030, ASP 1031, ASP 1033, GLU 1047, GLU 1051, ASN 1052, GLU 1066, and GLN 1122, or

wherein the clostridial toxin is BoNT/E and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 859, ASP 860, ASN 892, ASP 893, ASP 904, ASP 909, ASN 928, ASN 932, ASN 934, ASN 935, GLU 936, ASP 945, ASN 946, ASN 947, ASN 966, ASN 976, ASN 979, ASN 981, ASP 985, GLN 1014, ASN 1019, ASN 1022, ASP 1027, ASN 1035, and ASN 1140, or

wherein the clostridial toxin is BoNT/F and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 879, ASP 896, ASN 922, ASN 923, ASN 928, ASN 947, ASN 950, ASN 952, ASN 953, GLU 954, ASN 963, ASN 964, ASN 965, ASN 987, GLN 997, ASN 1037, ASP 1040, ASP 1045, ASN 1055, and ASP 1056, or

wherein the clostridial toxin is BoNT/G and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASP

900, ASN 909, ASN 910, GLU 912, ASN 913, ASN 945, ASN 947, GLU 956, ASN 965, ASP 966, ASN 986, ASN 1001, ASN 1038, ASP 1040, ASN 1046, ASP 1057, GLU 1073, ASN 1075, and ASN 1090, or

wherein the clostridial toxin is a tetanus neurotoxin (TeNT) and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 893, ASP 894, ASP 911, ASN 919, ASN 927, ASN 928, GLU 929, GLN 968, ASN 972, GLU 973, GLU 1010, ASP 1018, ASN 1079, ASN 1080, ASN 1081, and ASN 1097, and

wherein the at least four amino acid modifications are selected from:

i. substitution of an acidic amino acid residue with a basic amino acid residue;
ii. substitution of an acidic amino acid residue with an uncharged amino acid residue;
iii. substitution of an uncharged amino acid residue with a basic amino acid residue;
iv. insertion of a basic amino acid residue; or
v. deletion of an acidic amino acid residue, and

wherein the engineered clostridial toxin has a Safety Ratio of at least 8, wherein the Safety Ratio is calculated as: dose of toxin required for -10% bodyweight change measured as pg/mouse divided by DAS ED_{50} measured as pg/mouse, wherein ED_{50} = dose required to produce a DAS score of 2.

In another aspect, the present disclsoure refers to a method for increasing the isoelectric point (pI) of a clostridial toxin, the method comprising modifying a clostridial toxin receptor binding domain (H_C domain) of a clostridial toxin to introduce at least four amino acid modifications, thereby increasing the pI of the engineered clostridial toxin,

wherein the at least four amino acid modifications increase the pI of the clostridial toxin to a value that is at least 0.2 pI units higher than the pI of the otherwise identical clostridial toxin lacking the at least four amino acid modifications, and

wherein the clostridial toxin is BoNT/A and the at least four amino acid

modifications comprise modification of at least four 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, or

wherein the clostridial toxin is BoNT/B and the at least four amino acid modifications comprise modification of at least four amino acids selected from: ASN 873, ASN 874, GLU 892, ASP 895, ASN 906, ASP 940, ASN 948, GLU 949, ASN 958, ASN 959, ASN 979, ASN 990, GLU 993, ASP 994, GLU 997, ASN 1012, ASN 1019, ASP 1030, ASP 1047, ASP 1049, GLU 1065, GLU 1072, GLN 1176, GLU 1189, GLU 1252, and ASN 1273, or

wherein the clostridial toxin is BoNT/C_1 and the at least four amino acid modifications comprise modification of at least four amino acids selected from: ASN 881, ASP 898, GLU 916, GLU 927, ASN 952, ASN 964, ASN 965, ASN 984, GLU 985, ASP 986, ASP 996, ASN 1000, GLU 1036, ASN 1041, ASP 1062, ASP 1064, GLU 1079, and ASP 1081, or

wherein the clostridial toxin is BoNT/D and the at least four amino acid modifications comprise modification of at least four amino acids selected from: ASN 877, ASP 893, ASN 894, ASN 898, ASN 920, ASN 945, ASN 948, GLU 957, GLN 958, ASN 959, ASN 968, ASN 979, GLU 1030, ASP 1031, ASP 1033, GLU 1047, GLU 1051, ASN 1052, GLU 1066, and GLN 1122, or

wherein the clostridial toxin is BoNT/E and the at least four amino acid modifications comprise modification of at least four amino acids selected from: ASN 859, ASP 860, ASN 892, ASP 893, ASP 904, ASP 909, ASN 928, ASN 932, ASN 934, ASN 935, GLU 936, ASP 945, ASN 946, ASN 947, ASN 966, ASN 976, ASN 979, ASN 981, ASP 985, GLN 1014, ASN 1019, ASN 1022, ASP 1027, ASN 1035, and ASN 1140, or

wherein the clostridial toxin is BoNT/F and the at least four amino acid modifications comprise modification of at least four amino acids selected from: ASN 879, ASP 896, ASN 922, ASN 923, ASN 928, ASN 947, ASN 950, ASN 952, ASN

953, GLU 954, ASN 963, ASN 964, ASN 965, ASN 987, GLN 997, ASN 1037, ASP 1040, ASP 1045, ASN 1055, and ASP 1056, or

wherein the clostridial toxin is BoNT/G and the at least four amino acid modifications comprise modification of at least four amino acids selected from: ASP 900, ASN 909, ASN 910, GLU 912, ASN 913, ASN 945, ASN 947, GLU 956, ASN 965, ASP 966, ASN 986, ASN 1001, ASN 1038, ASP 1040, ASN 1046, ASP 1057, GLU 1073, ASN 1075, and ASN 1090, or

wherein the clostridial toxin is a tetanus neurotoxin (TeNT) and the at least four amino acid modifications comprise modification of at least four amino acids selected from: ASN 893, ASP 894, ASP 911, ASN 919, ASN 927, ASN 928, GLU 929, GLN 968, ASN 972, GLU 973, GLU 1010, ASP 1018, ASN 1079, ASN 1080, ASN 1081, and ASN 1097, and

wherein the at least four amino acid modifications are selected from:

i. substitution of an acidic amino acid residue with a basic amino acid residue;
ii. substitution of an acidic amino acid residue with an uncharged amino acid residue;
iii. substitution of an uncharged amino acid residue with a basic amino acid residue;
iv. insertion of a basic amino acid residue; or
v. deletion of an acidic amino acid residue, and

wherein the engineered clostridial toxin has a Safety Ratio of at least 8, wherein the Safety Ratio is calculated as: dose of toxin required for -10% bodyweight change measured as pg/mouse divided by DAS ED_{50} measured as pg/mouse, wherein ED_{50} = dose required to produce a DAS score of 2.

In yet another aspect, the present disclosure refers to an engineered clostridial toxin comprising SEQ ID NO: 2 that has at least four amino acid modifications located in the clostridial toxin receptor binding domain (H_C domain),

wherein the at least four amino acid modifications increase the isoelectric point (pI) of the engineered clostridial toxin to a value that is at least 0.2 pI units higher than

the pI of a clostridial toxin comprising SEQ ID NO: 2 lacking the at least four amino acid modifications,

wherein the at least four amino acid modifications consist of modification of at least four 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,

wherein the at least four amino acid modifications are selected from:

i. substitution of an acidic amino acid residue with a basic amino acid residue;
ii. substitution of an acidic amino acid residue with an uncharged amino acid residue;
iii. substitution of an uncharged amino acid residue with a basic amino acid residue;
iv. insertion of a basic amino acid residue; or
v. deletion of an acidic amino acid residue, and

wherein the engineered clostridial toxin has a Safety Ratio of at least 8, wherein the Safety Ratio is calculated as: dose of toxin required for -10% bodyweight change measured as pg/mouse divided by DAS ED_{50} measured as pg/mouse, wherein ED_{50} = dose required to produce a DAS score of 2.

In yet another aspect, the present disclosure refers to an engineered clostridial toxin comprising at least four amino acid modifications located in the clostridial toxin receptor binding domain (H_C domain),

wherein the at least four amino acid modifications increase the isoelectric point (pI) of the engineered clostridial to a value that is at least 0.2 pI units higher than the pI of the otherwise identical clostridial toxin lacking the at least four amino acid modifications, and

wherein the engineered clostridial toxin is BoNT/B and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 873, ASN 874, GLU 892, ASP 895, ASN 906, ASP 940, ASN 948, GLU 949,

ASN 958, ASN 959, ASN 979, ASN 990, GLU 993, ASP 994, GLU 997, ASN 1012, ASN 1019, ASP 1030, ASP 1047, ASP 1049, GLU 1065, GLU 1072, GLN 1176, GLU 1189, GLU 1252, and ASN 1273, or

wherein the engineered clostridial toxin is BoNT/C_1 and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 881, ASP 898, GLU 916, GLU 927, ASN 952, ASN 964, ASN 965, ASN 984, GLU 985, ASP 986, ASP 996, ASN 1000, GLU 1036, ASN 1041, ASP 1062, ASP 1064, GLU 1079, and ASP 1081, or

wherein the engineered clostridial toxin is BoNT/D and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 877, ASP 893, ASN 894, ASN 898, ASN 920, ASN 945, ASN 948, GLU 957, GLN 958, ASN 959, ASN 968, ASN 979, GLU 1030, ASP 1031, ASP 1033, GLU 1047, GLU 1051, ASN 1052, GLU 1066, and GLN 1122, or

wherein the engineered clostridial toxin is BoNT/E and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 859, ASP 860, ASN 892, ASP 893, ASP 904, ASP 909, ASN 928, ASN 932, ASN 934, ASN 935, GLU 936, ASP 945, ASN 946, ASN 947, ASN 966, ASN 976, ASN 979, ASN 981, ASP 985, GLN 1014, ASN 1019, ASN 1022, ASP 1027, ASN 1035, and ASN 1140, or

wherein the engineered clostridial toxin is BoNT/F and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 879, ASP 896, ASN 922, ASN 923, ASN 928, ASN 947, ASN 950, ASN 952, ASN 953, GLU 954, ASN 963, ASN 964, ASN 965, ASN 987, GLN 997, ASN 1037, ASP 1040, ASP 1045, ASN 1055, and ASP 1056, or

wherein the engineered clostridial toxin is BoNT/G and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASP 900, ASN 909, ASN 910, GLU 912, ASN 913, ASN 945, ASN 947, GLU 956, ASN 965, ASP 966, ASN 986, ASN 1001, ASN 1038, ASP 1040, ASN 1046, ASP 1057, GLU 1073, ASN 1075, and ASN 1090, or

wherein the engineered clostridial toxin is a tetanus neurotoxin (TeNT) and the at least four amino acid modifications consist of modification of at least four amino

acids selected from: ASN 893, ASP 894, ASP 911, ASN 919, ASN 927, ASN 928, GLU 929, GLN 968, ASN 972, GLU 973, GLU 1010, ASP 1018, ASN 1079, ASN 1080, ASN 1081, and ASN 1097, and

wherein the at least four amino acid modifications are selected from:

i. substitution of an acidic amino acid residue with a basic amino acid residue;
ii. substitution of an acidic amino acid residue with an uncharged amino acid residue;
iii. substitution of an uncharged amino acid residue with a basic amino acid residue;
iv. insertion of a basic amino acid residue; or
v. deletion of an acidic amino acid residue, and

wherein the engineered clostridial toxin has a Safety Ratio of at least 8, wherein the Safety Ratio is calculated as: dose of toxin required for -10% bodyweight change measured as pg/mouse divided by DAS ED_{50} measured as pg/mouse, wherein ED_{50} = dose required to produce a DAS score of 2.

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 protein 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 protein, and isolating the single-chain engineered clostridial toxin protein

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 protein obtainable by the method as disclosed herein, contacting the polypeptide with a protease that cleaves the polypeptide at a recognition site (cleavage site) located between the light chain and heavy chain, and converting the polypeptide into a di-chain polypeptide 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 an engineered clostridial toxin as disclosed herein or a di-chain engineered clostridial toxin as disclosed herein, for use in medicine.

In yet another aspect, the present disclosure refers to the use of an engineered clostridial toxin as disclosed herein or a di-chain engineered clostridial toxin as disclosed herein in the manufacture of a medicament for preventing or treating a disease or condition selected from: strabismus, blepharospasm, squint, dystonia, torticollis, neuromuscular disorder or condition of ocular motility, writer's cramp, bruxism, Wilson's disease, tremor, tics, segmental myoclonus, spasms, spasticity due to chronic multiple sclerosis, spasticity resulting in abnormal bladder control, animus, back spasm, charley horse, tension headaches, levator pelvic syndrome, spina bifida, tardive dyskinesia, Parkinson's disease, stuttering, hemifacial spasm, eyelid disorder, cerebral palsy, focal spasticity, spasmodic colitis, neurogenic bladder, anismus, limb spasticity, anal fissure, achalasia, dysphagia, lacrimation, hyperhydrosis, excessive salivation, excessive gastrointestinal secretions, muscle pain, headache pain, cancer, uterine disorders, uro-genital disorders, urogenital-neurological disorders, chronic neurogenic inflammation, and a smooth muscle disorder.

In yet another aspect, the present disclosure refers to the use of an engineered clostridial toxin as disclosed herein or a di-chain engineered clostridial toxin as disclosed herein in the manufacture of a medicament for cosmetic treatment benefitting from cell/muscle incapacitation.

In yet another aspect, the present disclosure refers to a method of cosmetic treatment benefitting from cell/muscle incapacitation comprising that an engineered clostridial toxin as disclosed herein or 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. An engineered clostridial toxin comprising between 4 and 40 amino acid modifications located in the clostridial toxin H_{CN} domain, wherein said between 4 and 40 amino acid modifications increase the isoelectric point (pI) of the engineered clostridial toxin to a value that is at least 0.2 pI units higher than the pI of an otherwise identical clostridial toxin lacking said between 4 and 40 amino acid modifications, wherein the engineered clostridial toxin is:

a. an engineered botulinum neurotoxin A (BoNT/A) having a pI of at least 6.6 and the between 4 and 40 amino acid modifications comprise modification of at least 4 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, and ASP 1086;

b. an engineered BoNT/B having a pI of at least 5.5 and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 873, ASN 874, GLU 892, ASP 895, ASN 906, ASP 940, ASN 948, GLU 949, ASN 958, ASN 959, ASN 979, ASN 990, GLU 993, ASP 994, GLU 997, ASN 1012, ASN 1019, ASP 1030, ASP 1047, ASP 1049, GLU 1065, and GLU 1072;

c. an engineered BoNT/C_1 having a pI of at least 5.7 and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 881, ASP 898, GLU 916, GLU 927, ASN 952, ASN 964, ASN 965, ASN 984, GLU 985, ASP 986, ASP 996, ASN 1000, GLU 1036, ASN 1041, ASP 1062, ASP 1064, GLU 1079, and ASP 1081;

d. an engineered BoNT/D having a pI of at least 5.7 and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 877, ASP 893, ASN 894, ASN 898, ASN 920, ASN 945, ASN 948, GLU 957, GLN 958, ASN 959, ASN 968, ASN 979, GLU 1030, ASP 1031, ASP 1033, GLU 1047, GLU 1051, ASN 1052, and GLU 1066;

e. an engineered BoNT/E having a pI of at least 6.2 and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 859, ASP 860, ASN 892, ASP 893, ASP 904, ASP 909, ASN 928, ASN 932, ASN 934, ASN 935, GLU 936, ASP 945, ASN 946, ASN 947, ASN 966, ASN 976, ASN 979, ASN 981, ASP 985, GLN 1014, ASN 1019, ASN 1022, ASP 1027, and ASN 1035;

f. an engineered BoNT/F having a pI of at least 5.8 and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 879, ASP 896, ASN 922, ASN 923, ASN 928, ASN 947, ASN 950, ASN 952, ASN 953, GLU 954, ASN 963, ASN 964, ASN 965, ASN 987, GLN 997, ASN 1037, ASP 1040, ASP 1045, ASN 1055, and ASP 1056;

g. an engineered BoNT/G having a pI of at least 5.4 and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASP 900, ASN 909, ASN 910, GLU 912, ASN 913, ASN 945, ASN 947, GLU 956, ASN 965, ASP 966, ASN 986, ASN 1001, ASN 1038, ASP 1040, ASN 1046, ASP 1057, GLU 1073, ASN 1075, and ASN 1090; or

h. an engineered tetanus neurotoxin (TeNT) having a pI of at least 6.0 and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 893, ASP 894, ASP 911, ASN 919, ASN 927, ASN 928, GLU 929, GLN 968, ASN 972, GLU 973, GLU 1010, ASP 1018, ASN 1079, ASN 1080, ASN 1081, and ASN 1097; and

wherein the between 4 and 40 amino acid modifications are selected from:

i. substitution of an acidic amino acid residue with a basic amino acid residue;

ii. substitution of an acidic amino acid residue with an uncharged amino acid residue;

iii. substitution of an uncharged amino acid residue with a basic amino acid residue;

iv. insertion of a basic amino acid residue; or

v. deletion of an acidic amino acid residue; and

wherein the engineered clostridial toxin has a Safety Ratio of at least 8, wherein the Safety Ratio is calculated as: dose of toxin required for -10% bodyweight change

measured as pg/mouse divided by DAS ED_{50} measured as pg/mouse, wherein ED_{50} = dose required to produce a DAS score of 2.

2. A method for producing an engineered clostridial toxin, the method comprising modifying an H_{CN} domain of a clostridial toxin to introduce between 4 and 40 amino acid modifications, thereby producing the engineered clostridial toxin,

wherein the between 4 and 40 amino acid modifications increase the isoelectric point (pI) of the engineered clostridial toxin to a value that is at least 0.2 pI units higher than the pI of an otherwise identical clostridial toxin lacking the between 4 and 40 amino acid modifications, and

wherein the clostridial toxin is a botulinum neurotoxin A (BoNT/A) and the between 4 and 40 amino acid modifications comprise modification of at least 4 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 1026, ASN 1032, ASN 1043, ASN 1046, ASN 1052, ASP 1058, HIS 1064, ASN 1080, GLU 1081, GLU 1083, and ASP 1086, or

wherein the clostridial toxin is a BoNT/B and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 873, ASN 874, GLU 892, ASP 895, ASN 906, ASP 940, ASN 948, GLU 949, ASN 958, ASN 959, ASN 979, ASN 990, GLU 993, ASP 994, GLU 997, ASN 1012, ASN 1019, ASP 1030, ASP 1047, ASP 1049, GLU 1065, and GLU 1072, or

wherein the clostridial toxin is a BoNT/C_1 and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 881, ASP 898, GLU 916, GLU 927, ASN 952, ASN 964, ASN 965, ASN 984, GLU 985, ASP 986, ASP 996, ASN 1000, GLU 1036, ASN 1041, ASP 1062, ASP 1064, GLU 1079, and ASP 1081, or

wherein the clostridial toxin is a BoNT/D and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 877, ASP 893, ASN 894, ASN 898, ASN 920, ASN 945, ASN 948, GLU 957, GLN 958, ASN 959, ASN 968, ASN 979, GLU 1030, ASP 1031, ASP 1033, GLU 1047, GLU 1051, ASN 1052, and GLU 1066, or

wherein the clostridial toxin is a BoNT/E and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 859, ASP 860, ASN 892, ASP 893, ASP 904, ASP 909, ASN 928, ASN 932, ASN 934, ASN 935, GLU

936, ASP 945, ASN 946, ASN 947, ASN 966, ASN 976, ASN 979, ASN 981, ASP 985, GLN 1014, ASN 1019, ASN 1022, ASP 1027, and ASN 1035, or

wherein the clostridial toxin is a BoNT/F and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 879, ASP 896, ASN 922, ASN 923, ASN 928, ASN 947, ASN 950, ASN 952, ASN 953, GLU 954, ASN 963, ASN 964, ASN 965, ASN 987, GLN 997, ASN 1037, ASP 1040, ASP 1045, ASN 1055, and ASP 1056, or

wherein the clostridial toxin is a BoNT/G and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASP 900, ASN 909, ASN 910, GLU 912, ASN 913, ASN 945, ASN 947, GLU 956, ASN 965, ASP 966, ASN 986, ASN 1001, ASN 1038, ASP 1040, ASN 1046, ASP 1057, GLU 1073, ASN 1075, and ASN 1090, or

wherein the clostridial toxin is a tetanus neurotoxin (TeNT) and the between 4 and 40 amino acid modifications comprise modification of at least 4 amino acids selected from: ASN 893, ASP 894, ASP 911, ASN 919, ASN 927, ASN 928, GLU 929, GLN 968, ASN 972, GLU 973, GLU 1010, ASP 1018, ASN 1079, ASN 1080, ASN 1081, and ASN 1097, and

wherein the between 4 and 40 amino acid modifications are selected from:

i. substitution of an acidic amino acid residue with a basic amino acid residue;
ii. substitution of an acidic amino acid residue with an uncharged amino acid residue;
iii. substitution of an uncharged amino acid residue with a basic amino acid residue;
iv. insertion of a basic amino acid residue; and
v. deletion of an acidic amino acid residue.

wherein the engineered clostridial toxin has a Safety Ratio of at least 8, wherein the Safety Ratio is calculated as: dose of toxin required for -10% bodyweight change measured as pg/mouse divided by DAS ED_{50} measured as pg/mouse, wherein ED_{50} = dose required to produce a DAS score of 2.

3. The method according to claim 2, wherein the between 4 and 40 amino acid modifications are introduced by:

a. synthesising a nucleic acid sequence that encodes the engineered clostridial toxin comprising the between 4 and 40 amino acid modifications; or
b. providing a DNA sequence encoding a clostridial toxin and modifying the DNA sequence to introduce the between 4 and 40 amino acid modifications into the encoded engineered clostridial toxin.

4. A method for producing an engineered clostridial toxin, the method comprising modifying a clostridial toxin receptor binding domain (H_C domain) of a clostridial toxin to introduce at least four amino acid modifications, thereby producing the engineered clostridial toxin,

wherein the at least four amino acid modifications increase the isoelectric point (pI) of the engineered clostridial toxin to a value that is at least 0.2 pI units higher than the pI of the otherwise identical clostridial toxin lacking the at least four amino acid modifications, and

wherein the clostridial toxin is botulinum neurotoxin A (BoNT/A) and the at least four amino acid modifications consist of modification of at least four 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, or

wherein the clostridial toxin is BoNT/B and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 873, ASN 874, GLU 892, ASP 895, ASN 906, ASP 940, ASN 948, GLU 949, ASN 958, ASN 959, ASN 979, ASN 990, GLU 993, ASP 994, GLU 997, ASN 1012, ASN 1019, ASP 1030, ASP 1047, ASP 1049, GLU 1065, GLU 1072, GLN 1176, GLU 1189, GLU 1252, and ASN 1273, or

wherein the clostridial toxin is BoNT/C_1 and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 881, ASP 898, GLU 916, GLU 927, ASN 952, ASN 964, ASN 965, ASN 984, GLU

985, ASP 986, ASP 996, ASN 1000, GLU 1036, ASN 1041, ASP 1062, ASP 1064, GLU 1079, and ASP 1081, or

wherein the clostridial toxin is BoNT/D and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 877, ASP 893, ASN 894, ASN 898, ASN 920, ASN 945, ASN 948, GLU 957, GLN 958, ASN 959, ASN 968, ASN 979, GLU 1030, ASP 1031, ASP 1033, GLU 1047, GLU 1051, ASN 1052, GLU 1066, and GLN 1122, or

wherein the clostridial toxin is BoNT/E and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 859, ASP 860, ASN 892, ASP 893, ASP 904, ASP 909, ASN 928, ASN 932, ASN 934, ASN 935, GLU 936, ASP 945, ASN 946, ASN 947, ASN 966, ASN 976, ASN 979, ASN 981, ASP 985, GLN 1014, ASN 1019, ASN 1022, ASP 1027, ASN 1035, and ASN 1140, or

wherein the clostridial toxin is BoNT/F and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 879, ASP 896, ASN 922, ASN 923, ASN 928, ASN 947, ASN 950, ASN 952, ASN 953, GLU 954, ASN 963, ASN 964, ASN 965, ASN 987, GLN 997, ASN 1037, ASP 1040, ASP 1045, ASN 1055, and ASP 1056, or

wherein the clostridial toxin is BoNT/G and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASP 900, ASN 909, ASN 910, GLU 912, ASN 913, ASN 945, ASN 947, GLU 956, ASN 965, ASP 966, ASN 986, ASN 1001, ASN 1038, ASP 1040, ASN 1046, ASP 1057, GLU 1073, ASN 1075, and ASN 1090, or

wherein the clostridial toxin is a tetanus neurotoxin (TeNT) and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 893, ASP 894, ASP 911, ASN 919, ASN 927, ASN 928, GLU 929, GLN 968, ASN 972, GLU 973, GLU 1010, ASP 1018, ASN 1079, ASN 1080, ASN 1081, and ASN 1097, and

wherein the at least four amino acid modifications are selected from:

i. substitution of an acidic amino acid residue with a basic amino acid residue;

ii. substitution of an acidic amino acid residue with an uncharged amino acid residue;

iii. substitution of an uncharged amino acid residue with a basic amino acid residue;

iv. insertion of a basic amino acid residue; or

v. deletion of an acidic amino acid residue, and

wherein the engineered clostridial toxin has a Safety Ratio of at least 8, wherein the Safety Ratio is calculated as: dose of toxin required for -10% bodyweight change measured as pg/mouse divided by DAS ED_{50} measured as pg/mouse, wherein ED_{50} = dose required to produce a DAS score of 2.

5. A method for increasing the isoelectric point (pI) of a clostridial toxin, the method comprising modifying a clostridial toxin receptor binding domain (H_C domain) of a clostridial toxin to introduce at least four amino acid modifications, thereby increasing the pI of the engineered clostridial toxin,

wherein the at least four amino acid modifications increase the pI of the clostridial toxin to a value that is at least 0.2 pI units higher than the pI of the otherwise identical clostridial toxin lacking the at least four amino acid modifications, and

wherein the clostridial toxin is BoNT/A and the at least four amino acid modifications comprise modification of at least four 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, or

wherein the clostridial toxin is BoNT/B and the at least four amino acid modifications comprise modification of at least four amino acids selected from: ASN 873, ASN 874, GLU 892, ASP 895, ASN 906, ASP 940, ASN 948, GLU 949, ASN 958, ASN 959, ASN 979, ASN 990, GLU 993, ASP 994, GLU 997, ASN 1012, ASN 1019, ASP 1030, ASP 1047, ASP 1049, GLU 1065, GLU 1072, GLN 1176, GLU 1189, GLU 1252, and ASN 1273, or

wherein the clostridial toxin is BoNT/C_1 and the at least four amino acid modifications comprise modification of at least four amino acids selected from: ASN 881, ASP 898, GLU 916, GLU 927, ASN 952, ASN 964, ASN 965, ASN 984, GLU 985, ASP 986, ASP 996, ASN 1000, GLU 1036, ASN 1041, ASP 1062, ASP 1064, GLU 1079, and ASP 1081, or

wherein the clostridial toxin is BoNT/D and the at least four amino acid modifications comprise modification of at least four amino acids selected from: ASN 877, ASP 893, ASN 894, ASN 898, ASN 920, ASN 945, ASN 948, GLU 957, GLN 958, ASN 959, ASN 968, ASN 979, GLU 1030, ASP 1031, ASP 1033, GLU 1047, GLU 1051, ASN 1052, GLU 1066, and GLN 1122, or

wherein the clostridial toxin is BoNT/E and the at least four amino acid modifications comprise modification of at least four amino acids selected from: ASN 859, ASP 860, ASN 892, ASP 893, ASP 904, ASP 909, ASN 928, ASN 932, ASN 934, ASN 935, GLU 936, ASP 945, ASN 946, ASN 947, ASN 966, ASN 976, ASN 979, ASN 981, ASP 985, GLN 1014, ASN 1019, ASN 1022, ASP 1027, ASN 1035, and ASN 1140, or

wherein the clostridial toxin is BoNT/F and the at least four amino acid modifications comprise modification of at least four amino acids selected from: ASN 879, ASP 896, ASN 922, ASN 923, ASN 928, ASN 947, ASN 950, ASN 952, ASN 953, GLU 954, ASN 963, ASN 964, ASN 965, ASN 987, GLN 997, ASN 1037, ASP 1040, ASP 1045, ASN 1055, and ASP 1056, or

wherein the clostridial toxin is BoNT/G and the at least four amino acid modifications comprise modification of at least four amino acids selected from: ASP 900, ASN 909, ASN 910, GLU 912, ASN 913, ASN 945, ASN 947, GLU 956, ASN 965, ASP 966, ASN 986, ASN 1001, ASN 1038, ASP 1040, ASN 1046, ASP 1057, GLU 1073, ASN 1075, and ASN 1090, or

wherein the clostridial toxin is a tetanus neurotoxin (TeNT) and the at least four amino acid modifications comprise modification of at least four amino acids selected from: ASN 893, ASP 894, ASP 911, ASN 919, ASN 927, ASN 928, GLU 929, GLN 968, ASN 972, GLU 973, GLU 1010, ASP 1018, ASN 1079, ASN 1080,

ASN 1081, and ASN 1097, and

wherein the at least four amino acid modifications are selected from:

i. substitution of an acidic amino acid residue with a basic amino acid residue;
ii. substitution of an acidic amino acid residue with an uncharged amino acid residue;
iii. substitution of an uncharged amino acid residue with a basic amino acid residue;
iv. insertion of a basic amino acid residue; or
v. deletion of an acidic amino acid residue, and

wherein the engineered clostridial toxin has a Safety Ratio of at least 8, wherein the Safety Ratio is calculated as: dose of toxin required for -10% bodyweight change measured as pg/mouse divided by DAS ED_{50} measured as pg/mouse, wherein ED_{50} = dose required to produce a DAS score of 2.

6. The method according to claim 4 or 5, wherein the at least four amino acid modifications are introduced by:

a. synthesising a nucleic acid sequence that encodes the engineered clostridial toxin comprising the at least four amino acid modifications; or
b. providing a DNA sequence encoding a clostridial toxin and modifying the DNA sequence to introduce the at least four amino acid modifications into the encoded engineered clostridial toxin.

7. The method according to claim 3 or 6, wherein the method further comprises expressing the synthesised nucleic acid or DNA sequence in a suitable host cell *in vitro*, lysing the host cell to provide a host cell homogenate containing the engineered clostridial toxin, and isolating the engineered clostridial toxin.

8. The engineered clostridial toxin or method according to any one of claims 1-7, wherein the engineered clostridial toxin comprises an amino acid sequence having at least 90% sequence identity to an amino acid sequence selected from SEQ ID NOs: 4, 6, or 8.

9. The engineered clostridial toxin or method according to any one of claims 1-8, wherein the engineered clostridial toxin comprises an amino acid sequence selected from SEQ ID NOs: 4, 6, or 8.

10. The engineered clostridial toxin or method according to any one of claims 1-9, further comprising at least one amino acid modification located in the H_{CC} domain.

11. The engineered clostridial toxin or method according to any one of claims 1-10, wherein the engineered clostridial toxin is an engineered BoNT/A comprising modification of the following seven amino acids: ASN 886, ASN 930, SER 955, GLN 991, ASN 1026, ASN 1052, and GLN 1229.

12. The engineered clostridial toxin or method according to any one of claims 1-10, wherein the engineered clostridial toxin is an engineered BoNT/A comprising modification of the following seven amino acids: ASN 930, ASN 954, SER 955, GLN 991, ASN 1026, ASN 1052, and GLN 1229.

13. The engineered clostridial toxin or method according to any one of claims 1-10, wherein the engineered clostridial toxin is an engineered BoNT/A comprising modification of the following seven amino acids: ASN 930, SER 955, GLN 991, ASN 1025, ASN 1026, ASN 1052, and GLN 1229.

14. The engineered clostridial toxin or method according to any one of claims 1-13, wherein the amino acid modifications increase the isoelectric point (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 amino acid modifications.

15. The engineered clostridial toxin or method according to any one of claims 1-14, wherein the amino acid modifications increase the isoelectric point (pI) of the engineered clostridial toxin to a value that is at least one pI unit higher than the pI of an otherwise identical clostridial toxin lacking said amino acid modifications.

16. The engineered clostridial toxin or method according to any one of claims 1-15, wherein said amino acid modifications increase the isoelectric point (pI) of the engineered clostridial toxin to a value that is at least two pI units higher than the pI of an otherwise identical clostridial toxin lacking said amino acid modifications.

17. The engineered clostridial toxin or method according to any one of claims 1-16, wherein said amino acid modifications increase the isoelectric point (pI) of the engineered clostridial toxin to a value that is between 2 and 5 pI units higher than the pI of an otherwise identical clostridial toxin lacking said amino acid modifications.

18. The engineered clostridial toxin or method according to any one of claims 1-17, wherein the engineered clostridial toxin has a pI of at least 6.

19. The engineered clostridial toxin or method according to any one of claims 1-18, wherein the engineered clostridial toxin has a pI of between 6 and 10.

20. The engineered clostridial toxin or method according to any one of claims 1-19, wherein the engineered clostridial toxin has a pI of between 7 and 9.

21. The engineered clostridial toxin or method according to any one of claims 1-20, wherein the engineered clostridial toxin has a pI of between 8 and 9.

22. The engineered clostridial toxin or method according to any one of claims 1-21, wherein the amino acid modifications comprise modification of an amino acid residue selected from: an aspartic acid residue, a glutamic acid residue, a histidine residue, a serine residue, a

threonine residue, an asparagine residue, a glutamine residue, a cysteine residue, or a tyrosine residue.

23. The engineered clostridial toxin or method according to claim 22, wherein the amino acid residue is substituted with a lysine residue or an arginine residue.

24. An engineered clostridial toxin comprising SEQ ID NO: 2 that has at least four amino acid modifications located in the clostridial toxin receptor binding domain (H_C domain),

wherein the at least four amino acid modifications increase the isoelectric point (pI) of the engineered clostridial toxin to a value that is at least 0.2 pI units higher than the pI of a clostridial toxin comprising SEQ ID NO: 2 lacking the at least four amino acid modifications,

wherein the at least four amino acid modifications consist of modification of at least four 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,

wherein the at least four amino acid modifications are selected from:

i. substitution of an acidic amino acid residue with a basic amino acid residue;
ii. substitution of an acidic amino acid residue with an uncharged amino acid residue;
iii. substitution of an uncharged amino acid residue with a basic amino acid residue;
iv. insertion of a basic amino acid residue; or
v. deletion of an acidic amino acid residue, and

wherein the engineered clostridial toxin has a Safety Ratio of at least 8, wherein the Safety Ratio is calculated as: dose of toxin required for -10% bodyweight change measured as pg/mouse divided by DAS ED_{50} measured as pg/mouse, wherein ED_{50} = dose required to produce a DAS score of 2.

25. The engineered clostridial toxin according to claim 24, wherein the engineered clostridial toxin:

a. is encoded by a nucleotide sequence comprising SEQ ID NO: 3 and/or comprises SEQ ID NO: 4; or

b. is encoded by a nucleotide sequence comprising SEQ ID NO: 5 and/or comprises SEQ ID NO: 6; or

c. is encoded by a nucleotide sequence comprising SEQ ID NO: 7 and/or comprises SEQ ID NO: 8; or

d. is encoded by a nucleotide sequence comprising SEQ ID NO: 9 and/or comprises SEQ ID NO: 10.

26. An engineered clostridial toxin comprising at least four amino acid modifications located in the clostridial toxin receptor binding domain (H_C domain),

wherein the at least four amino acid modifications increase the isoelectric point (pI) of the engineered clostridial to a value that is at least 0.2 pI units higher than the pI of the otherwise identical clostridial toxin lacking the at least four amino acid modifications, and

wherein the engineered clostridial toxin is BoNT/B and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 873, ASN 874, GLU 892, ASP 895, ASN 906, ASP 940, ASN 948, GLU 949, ASN 958, ASN 959, ASN 979, ASN 990, GLU 993, ASP 994, GLU 997, ASN 1012, ASN 1019, ASP 1030, ASP 1047, ASP 1049, GLU 1065, GLU 1072, GLN 1176, GLU 1189, GLU 1252, and ASN 1273, or

wherein the engineered clostridial toxin is BoNT/C_1 and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 881, ASP 898, GLU 916, GLU 927, ASN 952, ASN 964, ASN 965, ASN 984, GLU 985, ASP 986, ASP 996, ASN 1000, GLU 1036, ASN 1041, ASP 1062, ASP 1064, GLU 1079, and ASP 1081, or

wherein the engineered clostridial toxin is BoNT/D and the at least four amino acid modifications consist of modification of at least four amino acids selected from:

ASN 877, ASP 893, ASN 894, ASN 898, ASN 920, ASN 945, ASN 948, GLU 957, GLN 958, ASN 959, ASN 968, ASN 979, GLU 1030, ASP 1031, ASP 1033, GLU 1047, GLU 1051, ASN 1052, GLU 1066, and GLN 1122, or

wherein the engineered clostridial toxin is BoNT/E and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 859, ASP 860, ASN 892, ASP 893, ASP 904, ASP 909, ASN 928, ASN 932, ASN 934, ASN 935, GLU 936, ASP 945, ASN 946, ASN 947, ASN 966, ASN 976, ASN 979, ASN 981, ASP 985, GLN 1014, ASN 1019, ASN 1022, ASP 1027, ASN 1035, and ASN 1140, or

wherein the engineered clostridial toxin is BoNT/F and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 879, ASP 896, ASN 922, ASN 923, ASN 928, ASN 947, ASN 950, ASN 952, ASN 953, GLU 954, ASN 963, ASN 964, ASN 965, ASN 987, GLN 997, ASN 1037, ASP 1040, ASP 1045, ASN 1055, and ASP 1056, or

wherein the engineered clostridial toxin is BoNT/G and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASP 900, ASN 909, ASN 910, GLU 912, ASN 913, ASN 945, ASN 947, GLU 956, ASN 965, ASP 966, ASN 986, ASN 1001, ASN 1038, ASP 1040, ASN 1046, ASP 1057, GLU 1073, ASN 1075, and ASN 1090, or

wherein the engineered clostridial toxin is a tetanus neurotoxin (TeNT) and the at least four amino acid modifications consist of modification of at least four amino acids selected from: ASN 893, ASP 894, ASP 911, ASN 919, ASN 927, ASN 928, GLU 929, GLN 968, ASN 972, GLU 973, GLU 1010, ASP 1018, ASN 1079, ASN 1080, ASN 1081, and ASN 1097, and

wherein the at least four amino acid modifications are selected from:

i. substitution of an acidic amino acid residue with a basic amino acid residue;
ii. substitution of an acidic amino acid residue with an uncharged amino acid residue;
iii. substitution of an uncharged amino acid residue with a basic amino acid residue;

iv. insertion of a basic amino acid residue; or

v. deletion of an acidic amino acid residue, and

wherein the engineered clostridial toxin has a Safety Ratio of at least 8, wherein the Safety Ratio is calculated as: dose of toxin required for -10% bodyweight change measured as pg/mouse divided by DAS ED_{50} measured as pg/mouse, wherein ED_{50} = dose required to produce a DAS score of 2.

27. A nucleic acid comprising a nucleic acid sequence encoding an engineered clostridial toxin according to any one of claims 1 or 8-26.

28. An *in vitro* method of producing a single-chain engineered clostridial toxin protein having a light chain and a heavy chain, the method comprising expressing a nucleic acid according to claim 27 in a suitable host cell, lysing the host cell to provide a host cell homogenate containing the single-chain engineered clostridial toxin protein, and isolating the single-chain engineered clostridial toxin protein.

29. A method of activating an engineered clostridial toxin, the method comprising providing a single-chain engineered clostridial toxin protein obtainable by the method of claim 28, contacting the polypeptide with a protease that cleaves the polypeptide at a recognition site (cleavage site) located between the light chain and heavy chain, and converting the polypeptide into a di-chain polypeptide wherein the light chain and heavy chain are joined together by a disulphide bond.

30. A di-chain engineered clostridial toxin obtainable by the method of claim 29.

31. An engineered clostridial toxin according to any one of claims 1 or 8-26 or a di-chain engineered clostridial toxin according to claim 30, for use in medicine.

32. Use of an engineered clostridial toxin according to any one of claims 1 or 8-26 or a di-chain engineered clostridial toxin according to claim 30 in the manufacture of a medicament for preventing or treating a disease or condition selected from: strabismus, blepharospasm, squint, dystonia, torticollis, neuromuscular disorder or condition of ocular motility, writer's cramp, bruxism, Wilson's disease, tremor, tics, segmental myoclonus, spasms, spasticity due to chronic multiple sclerosis, spasticity resulting in abnormal bladder control, animus, back

spasm, charley horse, tension headaches, levator pelvic syndrome, spina bifida, tardive dyskinesia, Parkinson's disease, stuttering, hemifacial spasm, eyelid disorder, cerebral palsy, focal spasticity, spasmodic colitis, neurogenic bladder, anismus, limb spasticity, anal fissure, achalasia, dysphagia, lacrimation, hyperhydrosis, excessive salivation, excessive gastrointestinal secretions, muscle pain, headache pain, cancer, uterine disorders, uro-genital disorders, urogenital-neurological disorders, chronic neurogenic inflammation, and a smooth muscle disorder.

33. The use according to claim 32, wherein the dystonia is spasmodic dystonia, oromandibular dystonia, focal dystonia, tardive dystonia, laryngeal dystonia, limb dystonia, or cervical dystonia, or wherein the torticollis is spasmodic torticollis, or wherein the neuromuscular disorder or condition of ocular motility is concomitant strabismus, vertical strabismus, lateral rectus palsy, nystagmus, or dysthyroid myopathy, or wherein the muscle pain is pain from muscle spasms.34. Use of an engineered clostridial toxin according to any one of claims 1 or 8-26 or a di-chain engineered clostridial toxin according to claim 30 in the manufacture of a medicament for cosmetic treatment benefitting from cell/muscle incapacitation.

35. The use according to claim 34, wherein the cosmetic treatment comprises treating brow furrows and/or skin wrinkles.

36. A method of cosmetic treatment benefitting from cell/muscle incapacitation comprising that an engineered clostridial toxin according to any one of claims 1 or 8-26 or a di-chain engineered clostridial toxin according to claim 30 is to be administered.

37. The method according to claim 36, wherein the cosmetic treatment comprises treating brow furrows and/or skin wrinkles.