

WE CLAIM:

1. A cysteine engineered antibody construct comprising one or more cysteine insertion mutations selected from:

- (a) an insertion of a cysteine residue between positions 39 and 40 in a VL domain;
- (b) an insertion of a cysteine residue between positions 40 and 41 in a VL domain;
- (c) an insertion of a cysteine residue between positions 126 and 127 in a CL domain;
- (d) an insertion of a cysteine residue between positions 148 and 149 in a CL domain;
- (e) an insertion of a cysteine residue between positions 149 and 150 in a CL domain;
- (f) an insertion of a cysteine residue between positions 9 and 10 in a VH domain;
- (g) an insertion of a cysteine residue between positions 169 and 170 in a CH1 domain;
- (h) an insertion of a cysteine residue between positions 237 and 238 in a CH2 domain;
- (i) an insertion of a cysteine residue between positions 295 and 296 in a CH2 domain, and
- (j) an insertion of a cysteine residue between positions 299 and 300 in a CH2 domain,

wherein the numbering of amino acids in the VL, CL, VH and CH1 domains is Kabat numbering and the numbering of amino acids in the CH2 domain is EU numbering, and

wherein the antibody construct is based on an immunoglobulin G (IgG).

2. The cysteine engineered antibody construct according to claim 1, wherein the melting temperature (T_m) of the domain of the cysteine engineered antibody construct comprising the cysteine insertion mutation is within 0°C to 8°C of the T_m of the same domain in the corresponding parental antibody construct that lacks the cysteine insertion mutation.

3. The cysteine engineered antibody construct according to claim 1 or 2, wherein the antibody construct comprises one cysteine insertion mutation.

4. The cysteine engineered antibody construct according to claim 1 or 2, wherein the antibody construct comprises two cysteine insertion mutations.
5. The cysteine engineered antibody construct according to claim 4, wherein the two cysteine insertion mutations are symmetrical mutations.
6. The cysteine engineered antibody construct according to claim 1 or 2, wherein the antibody construct comprises three cysteine insertion mutations.
7. The cysteine engineered antibody construct according to claim 6, wherein two of the three cysteine insertion mutations are symmetrical mutations.
8. The cysteine engineered antibody construct according to any one of claims 3, 6 or 7, wherein each cysteine insertion mutation is independently selected from:
 - (a) an insertion of a cysteine residue between positions 9 and 10 in a VH domain;
 - (b) an insertion of a cysteine residue between positions 40 and 41 in a VL domain;
 - (c) an insertion of a cysteine residue between positions 237 and 238 in a CH2 domain, and
 - (d) an insertion of a cysteine residue between positions 299 and 300 in a CH2 domain.
9. The cysteine engineered antibody construct according to claim 3, wherein the cysteine insertion mutation is selected from:
 - (a) an insertion of a cysteine residue between positions 9 and 10 in a VH domain;
 - (b) an insertion of a cysteine residue between positions 237 and 238 in a CH2 domain, and
 - (c) an insertion of a cysteine residue between positions 299 and 300 in a CH2 domain.
10. The cysteine engineered antibody construct according to claim 1 or 2, wherein the antibody construct comprises four or six cysteine insertion mutations.
11. The cysteine engineered antibody construct according to claim 10, wherein the cysteine insertion mutations are selected from:

- (a) an insertion of a cysteine residue between positions 40 and 41 in a VL domain;
 - (b) an insertion of a cysteine residue between positions 126 and 127 in a CL domain;
 - (c) an insertion of a cysteine residue between positions 9 and 10 in a VH domain;
 - (d) an insertion of a cysteine residue between positions 237 and 238 in a CH2 domain, and
 - (e) an insertion of a cysteine residue between positions 299 and 300 in a CH2 domain.
12. The cysteine engineered antibody construct according to any one of claims 1 to 11, wherein the antibody construct comprises a VH domain or a VH domain and a VL domain.
13. The cysteine engineered antibody construct according to claim 12, wherein the antibody construct comprises one or more antigen-binding domains, at least one of the antigen-binding domains comprising the VH domain or the VH domain and the VL domain.
14. The cysteine engineered antibody construct according to claim 13, wherein the antibody construct comprises two antigen-binding domains.
15. The cysteine engineered antibody construct according to claim 14, wherein the antibody construct is bispecific.
16. The cysteine engineered antibody construct according to any one of claims 13 to 15, wherein at least one of the antigen-binding domains binds to a tumor-associated antigen.
17. The cysteine engineered antibody construct according to any one of claims 1 to 16, wherein the antibody construct comprises an Fc region.
18. The cysteine engineered antibody construct according to any one of claims 1 to 17, wherein the antibody construct is based on an IgG1.
19. The cysteine engineered antibody construct according to any one of claims 1 to 18, wherein the IgG is a human IgG.
20. The cysteine engineered antibody construct according to any one of claims 1 to 19, wherein the antibody construct comprises a heterodimeric Fc region.

21. The cysteine engineered antibody construct according to claim 20, wherein the heterodimeric Fc region comprises a modified CH3 domain comprising amino acid mutations that promote formation of the heterodimeric Fc over a homodimeric Fc.

22. A conjugate comprising the cysteine engineered antibody construct according to any one of claims 1 to 21, and one or more active agents conjugated to each of the one or more inserted cysteine residues.

23. A conjugate having Formula (I):



wherein:

A is a cysteine engineered antibody construct;

L is a linker;

D is an active agent;

q is an integer between 1 and 4, and

p is an integer between 1 and 8,

wherein the cysteine engineered antibody construct comprises one or more cysteine insertion mutations selected from:

- (a) an insertion of a cysteine residue between positions 39 and 40 in a VL domain;
- (b) an insertion of a cysteine residue between positions 40 and 41 in a VL domain;
- (c) an insertion of a cysteine residue between positions 126 and 127 in a CL domain;
- (d) an insertion of a cysteine residue between positions 148 and 149 in a CL domain;
- (e) an insertion of a cysteine residue between positions 149 and 150 in a CL domain;
- (f) an insertion of a cysteine residue between positions 9 and 10 in a VH domain;

(g) an insertion of a cysteine residue between positions 169 and 170 in a CH1 domain;

(h) an insertion of a cysteine residue between positions 237 and 238 in a CH2 domain;

(i) an insertion of a cysteine residue between positions 295 and 296 in a CH2 domain, and

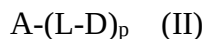
(j) an insertion of a cysteine residue between positions 299 and 300 in a CH2 domain,

wherein the numbering of amino acids in the VL, CL, VH and CH1 domains is Kabat numbering and the numbering of amino acids in the CH2 domain is EU numbering,

wherein the cysteine engineered antibody construct is based on an immunoglobulin G (IgG), and

wherein each D is linked to an inserted cysteine residue via L.

24. The conjugate of claim 23, wherein the conjugate has Formula (II):



wherein:

A is the cysteine engineered antibody construct;

L is the linker;

D is the active agent, and

p is an integer between 1 and 8.

25. The conjugate according to any one of claims 22 to 24, wherein the active agent is a diagnostic agent or a labelling agent.

26. The conjugate according to any one of claims 22 to 24, wherein the active agent is a therapeutic agent.

27. A composition comprising the conjugate according to any one of claims 22 to 26, and a pharmaceutically acceptable carrier or diluent.

28. A conjugate according to claim 26 for use in therapy.
29. Use of the conjugate according to claim 26 in the manufacture of a medicament for the treatment of a subject in need thereof.
30. A method of preparing the conjugate according to any one of claims 22 to 26 comprising submitting the cysteine engineered antibody construct to reducing conditions such that the thiol group of the one or more inserted cysteine residues is reduced, and reacting a thiol reactive linker-active agent with the antibody construct under conditions that permit formation of a bond between the linker and the reduced thiol.
31. A method of preparing an antibody-drug conjugate having a pre-determined drug-to-antibody ratio (DAR), the method comprising:
- (i) providing a cysteine engineered antibody construct comprising one or more cysteine insertion mutations selected from:
 - (a) an insertion of a cysteine residue between positions 39 and 40 in a VL domain;
 - (b) an insertion of a cysteine residue between positions 40 and 41 in a VL domain;
 - (c) an insertion of a cysteine residue between positions 126 and 127 in a CL domain;
 - (d) an insertion of a cysteine residue between positions 148 and 149 in a CL domain;
 - (e) an insertion of a cysteine residue between positions 149 and 150 in a CL domain;
 - (f) an insertion of a cysteine residue between positions 9 and 10 in a VH domain;
 - (g) an insertion of a cysteine residue between positions 169 and 170 in a CH1 domain;
 - (h) an insertion of a cysteine residue between positions 237 and 238 in a CH2 domain;
 - (i) an insertion of a cysteine residue between positions 295 and 296 in a CH2 domain,
- and

(j) an insertion of a cysteine residue between positions 299 and 300 in a CH2 domain,
and

(ii) reacting the cysteine engineered antibody construct with a drug-linker to provide the antibody-drug conjugate;

wherein the pre-determined DAR is 1, 2, 3, 4, 5, 6, 7 or 8, and the cysteine engineered antibody construct comprises the same number of cysteine insertion mutations as the pre-determined DAR,

wherein the numbering of amino acids in the VL, CL, VH and CH1 domains is Kabat numbering and the numbering of amino acids in the CH2 domain is EU numbering, and

wherein the cysteine engineered antibody construct is based on an immunoglobulin G (IgG).

32. The method according to claim 31, wherein the pre-determined DAR is 2.

33. The method according to claim 31, wherein the pre-determined DAR is 1 or 3.

34. The method according to claim 33, wherein the cysteine insertion mutations are selected from:

(a) an insertion of a cysteine residue between positions 9 and 10 in a VH domain;

(b) an insertion of a cysteine residue between positions 40 and 41 in a VL domain;

(c) an insertion of a cysteine residue between positions 237 and 238 in a CH2 domain, and

(d) an insertion of a cysteine residue between positions 299 and 300 in a CH2 domain.

35. The method according to claim 31, wherein the pre-determined DAR is 4 or 6.

36. The method according to claim 35, wherein the cysteine insertion mutations are selected from:

(a) an insertion of a cysteine residue between positions 40 and 41 in a VL domain;

- (b) an insertion of a cysteine residue between positions 126 and 127 in a CL domain;
 - (c) an insertion of a cysteine residue between positions 9 and 10 in a VH domain;
 - (d) an insertion of a cysteine residue between positions 237 and 238 in a CH2 domain, and
 - (e) an insertion of a cysteine residue between positions 299 and 300 in a CH2 domain.
37. A polynucleotide or set of polynucleotides encoding the cysteine engineered antibody construct according to any one of claims 1 to 21.
38. A vector comprising one or more polynucleotides encoding the cysteine engineered antibody construct according to any one of claims 1 to 21.
39. A host cell comprising the vector according to claim 38.