

CLAIMS

1. A method for generating a conjugated immunoglobulin, the method comprising:
 - (1) incubating an immunoglobulin with a microbial transglutaminase and a functional agent comprising an acyl donor substrate,
 - a) wherein the immunoglobulin comprises at least one amino acid residue but less than 13 amino acid residues after a Lysine 447 (K447) on a heavy chain of the immunoglobulin, wherein the amino acid residues are numbered according to the EU numbering system,
 - i) wherein, when the immunoglobulin comprises one amino acid residue after the C-terminal lysine, the one amino acid residue after the C-terminal lysine is glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tyrosine, tryptophan, serine, threonine, cysteine, asparagine, glutamine, or histidine,
 - ii) wherein, when the immunoglobulin comprises two amino acid residues after the C-terminal lysine, comprising a first amino acid residue after the C-terminal lysine and a second amino acid residue after the C-terminal lysine, the first amino acid residue after the C-terminal lysine is any amino acid residue except aspartic acid, glutamic acid, or proline; and the second amino acid residue after the C-terminal lysine is selected from the group consisting of phenylalanine, leucine, isoleucine, methionine, valine, serine, proline, threonine, alanine, tyrosine, histidine, glutamine, asparagine, aspartic acid, glutamic acid, cysteine, tryptophan, and glycine,
 - iii) wherein, when the immunoglobulin comprises three amino acid residues after the C-terminal lysine, comprising a first amino acid residue after the C-terminal lysine, a second amino acid residue after the C-terminal lysine, and a third amino acid residue after the C-terminal lysine, the third amino acid residue after the C-terminal lysine is selected from the group consisting of phenylalanine, leucine, isoleucine, methionine, valine, serine, proline, threonine, alanine, tyrosine, histidine, glutamine, asparagine, aspartic acid, glutamic acid, cysteine, tryptophan, and glycine,
 - iv) wherein, when the immunoglobulin comprises four amino acid residues after the C-terminal lysine, comprising a first amino acid residue after the C-terminal lysine, a second amino acid residue after the C-terminal lysine, a third

amino acid residue after the C-terminal lysine, and a fourth amino acid residue after the C-terminal lysine, the fourth amino acid residue after the C-terminal lysine is selected from the group consisting of phenylalanine, leucine, isoleucine, methionine, valine, serine, proline, threonine, alanine, tyrosine, histidine, glutamine, asparagine, aspartic acid, glutamic acid, cysteine, tryptophan, and glycine,

v) wherein, when the immunoglobulin comprises five amino acid residues after the C-terminal lysine, comprising a first amino acid residue after the C-terminal lysine, a second amino acid residue after the C-terminal lysine, a third amino acid residue after the C-terminal lysine, a fourth amino acid residue after the C-terminal lysine, and a fifth amino acid residue after the C-terminal lysine, the fifth amino acid residue after the C-terminal lysine is selected from the group consisting of phenylalanine, leucine, isoleucine, methionine, valine, serine, proline, threonine, alanine, tyrosine, histidine, glutamine, asparagine, aspartic acid, glutamic acid, cysteine, tryptophan, and glycine, or

vi) wherein, when the immunoglobulin comprises less than 13 amino acid residues after the C-terminal lysine, the last amino acid residue after the C-terminal lysine is selected from the group consisting of phenylalanine, leucine, isoleucine, methionine, valine, serine, proline, threonine, alanine, tyrosine, histidine, glutamine, asparagine, aspartic acid, glutamic acid, cysteine, tryptophan, and glycine,

b) wherein the acyl donor substrate comprises a glutamine residue, and

c) wherein the functional agent is a therapeutic agent or a diagnostic agent,

wherein the microbial transglutaminase conjugates K447 to the glutamine residue of the acyl donor substrate on the functional agent, or

(2) i) incubating an immunoglobulin with a microbial transglutaminase and an acyl donor substrate,

a) wherein the immunoglobulin comprises at least one amino acid but less than 13 amino acid residues residue after Lysine 447 (K447) on a heavy chain of the immunoglobulin,

I) wherein, when the immunoglobulin comprises one amino acid residue after the C-terminal lysine, the one amino acid residue after the C-terminal lysine is glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine,

tyrosine, tryptophan, serine, threonine, cysteine, asparagine, glutamine, or histidine,

II) wherein, when the immunoglobulin comprises two amino acid residues after the C-terminal lysine, comprising a first amino acid residue after the C-terminal lysine and a second amino acid residue after the C-terminal lysine, the first amino acid residue after the C-terminal lysine is any amino acid residue except aspartic acid, glutamic acid, or proline; and the second amino acid residue after the C-terminal lysine is selected from the group consisting of phenylalanine, leucine, isoleucine, methionine, valine, serine, proline, threonine, alanine, tyrosine, histidine, glutamine, asparagine, aspartic acid, glutamic acid, cysteine, tryptophan, and glycine,

III) wherein, when the immunoglobulin comprises three amino acid residues after the C-terminal lysine, comprising a first amino acid residue after the C-terminal lysine, a second amino acid residue after the C-terminal lysine, and a third amino acid residue after the C-terminal lysine, the third amino acid residue after the C-terminal lysine is selected from the group consisting of phenylalanine, leucine, isoleucine, methionine, valine, serine, proline, threonine, alanine, tyrosine, histidine, glutamine, asparagine, aspartic acid, glutamic acid, cysteine, tryptophan, and glycine,

IV) wherein, when the immunoglobulin comprises four amino acid residues after the C-terminal lysine, comprising a first amino acid residue after the C-terminal lysine, a second amino acid residue after the C-terminal lysine, a third amino acid residue after the C-terminal lysine, and a fourth amino acid residue after the C-terminal lysine, the fourth amino acid residue after the C-terminal lysine is selected from the group consisting of phenylalanine, leucine, isoleucine, methionine, valine, serine, proline, threonine, alanine, tyrosine, histidine, glutamine, asparagine, aspartic acid, glutamic acid, cysteine, tryptophan, and glycine,

V) wherein, when the immunoglobulin comprises five amino acid residues after the C-terminal lysine, comprising a first amino acid residue after the C-terminal lysine, a second amino acid residue after the C-terminal lysine, a third amino acid residue after the C-terminal lysine, a fourth amino acid residue after the C-terminal lysine, and a fifth amino acid residue after the C-terminal lysine, the fifth amino acid residue after the C-terminal lysine is selected from the group

consisting of phenylalanine, leucine, isoleucine, methionine, valine, serine, proline, threonine, alanine, tyrosine, histidine, glutamine, asparagine, aspartic acid, glutamic acid, cysteine, tryptophan, and glycine, or

VI) wherein, when the immunoglobulin comprises less than 13 amino acid residues after the C-terminal lysine, the last amino acid residue after the C-terminal lysine is selected from the group consisting of phenylalanine, leucine, isoleucine, methionine, valine, serine, proline, threonine, alanine, tyrosine, histidine, glutamine, asparagine, aspartic acid, glutamic acid, cysteine, tryptophan, and glycine, and

b) wherein the acyl donor substrate comprises a glutamine residue and a reactive group,

wherein the microbial transglutaminase conjugates K447 to the glutamine residue of the acyl donor substrate, and

ii) conjugating a functional agent to the reactive group of the acyl donor substrate, wherein the functional agent is a therapeutic agent or a diagnostic agent; optionally wherein the reactive group of the acyl donor substrate is conjugated to the functional agent by click chemistry,

thereby generating the conjugated immunoglobulin.

2. A conjugated immunoglobulin comprising an immunoglobulin and a functional agent, wherein

a) the immunoglobulin comprises at least one amino acid residue but less than 13 amino acid residues after Lysine 447 (K447) on a heavy chain of the immunoglobulin, wherein the amino acid residues are numbered according to the EU numbering system,

i) wherein, when the immunoglobulin comprises one amino acid residue after the C-terminal lysine, the one amino acid residue after the C-terminal lysine is glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tyrosine, tryptophan, serine, threonine, cysteine, asparagine, glutamine, or histidine,

ii) wherein, when the immunoglobulin comprises two amino acid residues after the C-terminal lysine, comprising a first amino acid residue after the C-terminal lysine and a second amino acid residue after the C-terminal lysine, the first amino acid residue after the C-terminal lysine is any amino acid residue except aspartic acid, glutamic acid, or proline; and the second amino acid residue after the C-terminal lysine is selected from the group consisting of phenylalanine, leucine, isoleucine, methionine, valine, serine,

proline, threonine, alanine, tyrosine, histidine, glutamine, asparagine, aspartic acid, glutamic acid, cysteine, tryptophan, and glycine,

iii) wherein, when the immunoglobulin comprises three amino acid residues after the C-terminal lysine, comprising a first amino acid residue after the C-terminal lysine, a second amino acid residue after the C-terminal lysine, and a third amino acid residue after the C-terminal lysine, the third amino acid residue after the C-terminal lysine is selected from the group consisting of phenylalanine, leucine, isoleucine, methionine, valine, serine, proline, threonine, alanine, tyrosine, histidine, glutamine, asparagine, aspartic acid, glutamic acid, cysteine, tryptophan, and glycine,

iv) wherein, when the immunoglobulin comprises four amino acid residues after the C-terminal lysine, comprising a first amino acid residue after the C-terminal lysine, a second amino acid residue after the C-terminal lysine, a third amino acid residue after the C-terminal lysine, and a fourth amino acid residue after the C-terminal lysine, the fourth amino acid residue after the C-terminal lysine is selected from the group consisting of phenylalanine, leucine, isoleucine, methionine, valine, serine, proline, threonine, alanine, tyrosine, histidine, glutamine, asparagine, aspartic acid, glutamic acid, cysteine, tryptophan, and glycine,

v) wherein, when the immunoglobulin comprises five amino acid residues after the C-terminal lysine, comprising a first amino acid residue after the C-terminal lysine, a second amino acid residue after the C-terminal lysine, a third amino acid residue after the C-terminal lysine, a fourth amino acid residue after the C-terminal lysine, and a fifth amino acid residue after the C-terminal lysine, the fifth amino acid residue after the C-terminal lysine is selected from the group consisting of phenylalanine, leucine, isoleucine, methionine, valine, serine, proline, threonine, alanine, tyrosine, histidine, glutamine, asparagine, aspartic acid, glutamic acid, cysteine, tryptophan, and glycine, or

vi) wherein, when the immunoglobulin comprises less than 13 amino acid residues after the C-terminal lysine, the last amino acid residue after the C-terminal lysine is selected from the group consisting of phenylalanine, leucine, isoleucine, methionine, valine, serine, proline, threonine, alanine, tyrosine, histidine, glutamine, asparagine, aspartic acid, glutamic acid, cysteine, tryptophan, and glycine, and

b) the functional agent comprises an acyl donor substrate, wherein the acyl donor substrate comprises a glutamine residue, and

c) the functional agent is a therapeutic agent or a diagnostic agent,

wherein K447 is conjugated to the glutamine residue of the acyl donor substrate; or

wherein

a) the immunoglobulin comprises at least one amino acid residue but less than 13 amino acids after Lysine 447 (K447) on a heavy chain of the immunoglobulin,

i) wherein, when the immunoglobulin comprises one amino acid residue after the C-terminal lysine, the one amino acid residue after the C-terminal lysine is glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tyrosine, tryptophan, serine, threonine, cysteine, asparagine, glutamine, or histidine,

ii) wherein, when the immunoglobulin comprises two amino acid residues after the C-terminal lysine, comprising a first amino acid residue after the C-terminal lysine and a second amino acid residue after the C-terminal lysine, the first amino acid residue after the C-terminal lysine is any amino acid residue except aspartic acid, glutamic acid, or proline; and the second amino acid residue after the C-terminal lysine is selected from the group consisting of phenylalanine, leucine, isoleucine, methionine, valine, serine, proline, threonine, alanine, tyrosine, histidine, glutamine, asparagine, aspartic acid, glutamic acid, cysteine, tryptophan, and glycine,

iii) wherein, when the immunoglobulin comprises three amino acid residues after the C-terminal lysine, comprising a first amino acid residue after the C-terminal lysine, a second amino acid residue after the C-terminal lysine, and a third amino acid residue after the C-terminal lysine, the third amino acid residue after the C-terminal lysine is selected from the group consisting of phenylalanine, leucine, isoleucine, methionine, valine, serine, proline, threonine, alanine, tyrosine, histidine, glutamine, asparagine, aspartic acid, glutamic acid, cysteine, tryptophan, and glycine,

iv) wherein, when the immunoglobulin comprises four amino acid residues after the C-terminal lysine, comprising a first amino acid residue after the C-terminal lysine, a second amino acid residue after the C-terminal lysine, a third amino acid residue after the C-terminal lysine, and a fourth amino acid residue after the C-terminal lysine, the fourth amino acid residue after the C-terminal lysine is selected from the group consisting of phenylalanine, leucine, isoleucine, methionine, valine, serine, proline, threonine, alanine, tyrosine, histidine, glutamine, asparagine, aspartic acid, glutamic acid, cysteine, tryptophan, and glycine,

v) wherein, when the immunoglobulin comprises five amino acid residues after the C-terminal lysine, comprising a first amino acid residue after the C-terminal lysine, a second amino acid residue after the C-terminal lysine, a third amino acid residue after the C-terminal lysine, a fourth amino acid residue after the C-terminal lysine, and a fifth

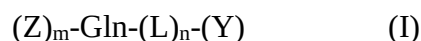
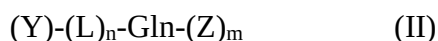
amino acid residue after the C-terminal lysine, the fifth amino acid residue after the C-terminal lysine is selected from the group consisting of phenylalanine, leucine, isoleucine, methionine, valine, serine, proline, threonine, alanine, tyrosine, histidine, glutamine, asparagine, aspartic acid, glutamic acid, cysteine, tryptophan, and glycine, or

vi) wherein, when the immunoglobulin comprises less than 13 amino acid residues after the C-terminal lysine, the last amino acid residue after the C-terminal lysine is selected from the group consisting of phenylalanine, leucine, isoleucine, methionine, valine, serine, proline, threonine, alanine, tyrosine, histidine, glutamine, asparagine, aspartic acid, glutamic acid, cysteine, tryptophan, and glycine,

b) K447 is conjugated to a glutamine residue on an acyl donor substrate, wherein the acyl donor substrate further comprises a reactive group, and

c) the reactive group is conjugated to a functional agent, wherein the functional agent is a therapeutic agent or a diagnostic agent.

3. The method of claim 1 or conjugated immunoglobulin of claim 2, wherein the functional agent comprising the acyl donor substrate is according to one Formulae (I) or (II):



wherein

Z is a carboxylbenzyloxy (CBZ) group or an amino acid residue;

Gln is a glutamine amino acid residue;

each L is independently a straight or branched linker from 1 to 20 carbon atoms, wherein one or more of the carbon atoms may be optionally and independently replaced with a nitrogen, oxygen or sulfur atom, and wherein each carbon and nitrogen atom may be optionally substituted; or each L is optionally and independently an amino acid residue;

m is an integer from 0 to 5;

n is an integer from 0 to 5; and

Y is a functional agent;

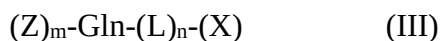
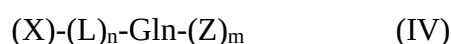
optionally wherein the functional agent comprising the acyl donor substrate is:

(a) according to formula (I), and wherein Z is a CBZ group; wherein each L is independently a polyethylene glycol moiety (PEG) (-O((CH₂)₂)-), ethyl amine (-NH((CH₂)₂)-) or propyl amine (-NH((CH₂)₃)-); and wherein n is 0, 1, 2, 3, 4 or 5; or

(b) according to formula (I), wherein Z is a CBZ group, and L is an amino acid; optionally wherein L is Gly; m is 1; and n is 0 or 1;

(c) according to formula (II), wherein Z is a CBZ group; m is 1; n is 1, 2 or 3; and at least one L is Gly.

4. The method of claim 1 or conjugated immunoglobulin of claim 2, wherein the acyl donor substrate is according to one Formulae (III) or (IV):



wherein

Z is a carboxylbenzyloxy (CBZ) group or an amino acid residue;

Gln is a glutamine amino acid residue;

each L is independently a straight or branched linker from 1 to 20 carbon atoms, wherein one or more of the carbon atoms may be optionally and independently replaced with a nitrogen, oxygen or sulfur atom, and wherein each carbon and nitrogen atom may be optionally substituted; or each L is optionally and independently an amino acid residue;

m is an integer from 0 to 5;

n is an integer from 0 to 5; and

X is a reactive group;

optionally wherein

(a) the acyl donor substrate is according to formula (III), and wherein Z is a CBZ group; wherein each L is independently a polyethylene glycol moiety (PEG) (-O((CH₂)₂)-), ethyl amine (-NH((CH₂)₂)-) or propyl amine (-NH((CH₂)₃)-); and wherein n is 0, 1, 2, 3, 4, or 5; or

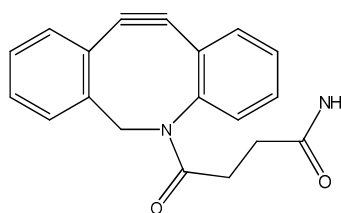
(b) the acyl donor substrate is according to formula (III), wherein Z is a CBZ group, and wherein one or more L is an amino acid; optionally wherein

(i) L is Gly; m is 1; and n is 1; or

(ii) m is 1; and n is 0; or

(c) the acyl donor substrate is according to formula (IV), wherein Z is a CBZ group; m is 1; n is 1, 2 or 3; and at least one L is Gly; or

(d) X is a reactive group selected from the group consisting of (1R,8S,9s)-



bicyclo[6.1.0]non-4-yn-9-ylmethanol (BCN),

(DBCO), trans-

cyclooctene (TCO), azido (N₃), alkyne, tetrazine methylcyclopropene, norbornene, hydrazide/hydrazine, and aldehyde.

5. The method of claim 1 or conjugated immunoglobulin of claim 2, wherein
(a) the therapeutic agent is an antibody or antigen-binding portion thereof, a chemotherapeutic agent, a drug agent, a radioactive agent, a cytotoxic agent, an antibiotic, a small molecule, a nucleic acid, or a polypeptide; or
(b) the diagnostic agent is a fluorophore, a fluorescent dye, a radionuclide, or an enzyme.
6. The method of claim 1 or conjugated immunoglobulin of claim 2, wherein the microbial transglutaminase is a *Streptomyces mobarensis* microbial transglutaminase.
7. The method of claim 1 or conjugated immunoglobulin of claim 2, wherein the immunoglobulin is (a) an IgG₁ immunoglobulin; or (b) an IgG₂, IgG₃, or IgG₄ immunoglobulin; or (c) an IgA₁, an IgA₂, or an IgM immunoglobulin which does not comprise a tailpiece; or (d) an IgD or IgE, immunoglobulin.
8. The method of any one of claims 1, and 3-7 or conjugated immunoglobulin of any one of claims 2, and 3-7, wherein the immunoglobulin
(a) is a human immunoglobulin or a humanized immunoglobulin; and/or
(b) is a chimeric immunoglobulin or a non-human immunoglobulin; and/or
(c) comprises two heavy chains and two light chains; optionally wherein there is no cross-linking between the two heavy chains of the immunoglobulin.
9. The method of any one of claims 1, and 3-7 or conjugated immunoglobulin of any one of claims 2, and 3-7, wherein the ratio of functional agent to immunoglobulin is 1:1 to 2:1.
10. The conjugated immunoglobulin of claim 2, wherein the functional agent is an antibody, or antigen-binding portion thereof, and wherein the immunoglobulin and the functional agent bind the same antigen or bind different antigens.
11. A pharmaceutical composition comprising the conjugated immunoglobulin of any one of claims 2, and 3-10 and a pharmaceutically acceptable carrier.
12. A conjugated immunoglobulin produced by the method of any one of claims 1, and 3-9.