[13] The present disclosure provides a new method for generating conditionally active polypeptides, taking advantage of directed mutagenesis for more efficiently production. The present disclosure also provides new conditionally active polypeptides produced by this method.

SUMMARY

[14] Disclosed is a conditionally active polypeptide obtainable by increasing a net charge of a parent polypeptide, wherein said conditionally active polypeptide exhibits a decrease in activity in a first assay at a first value of a condition compared to the same activity in a second assay at a second value of the same condition. Charged amino acid residues may be selected from Aspartic acid, Glutamic acid, Arginine, Lysine, and, optionally, Histidine.

[15] In each of the foregoing examples, the net charge may be increased through one or more techniques selected from increasing a total number of the charged amino acid residues in the polypeptide, decreasing a total number of uncharged amino acid residues in the polypeptide or a combination thereof. In each of the foregoing examples, the condition may be selected from pH, temperature, osmotic pressure, osmolality, oxidative stress, electrolyte concentration, concentration of a protein and combinations of two or more of these.

[16] In each of the foregoing examples, the condition may be pH, and the conditionally active polypeptide may exhibit the decreased activity at a normal physiological pH and the increased activity at an aberrant pH that differs from the normal physiological pH.

[17] In each of the foregoing examples, the conditional activity may be assayed in the presence in the presence of at least one species having a molecular weight of less than 900 a.m.u. and a pKa up to 2 units, or up to 3 units away from the pH at which the increased activity is desired. In the foregoing examples, the pKa of the species may be between the first value of the pH and the second value of the pH.

[18] In each of the foregoing examples, the conditional activity may be assayed in the presence in the presence of at least one species selected from, histidine, histamine, hydrogenated adenosine diphosphate, hydrogen sulfide, hydrogenate adenosine triphosphate, citrate, acetate, lactate, bisulfide, ammonium, bicarbonate, dihydrogen phosphate and combinations thereof.

[19] In each of the forgoing examples, the first assay and the second assay may be conducted in assay solutions in the absence of serum.

[20] In each of the forgoing examples, the net charge may be increased by introducing one or more charged amino residues into the parent polypeptide through amino acid residue substitutions, amino acid insertions, or a combination thereof.

[21] In each of the forgoing examples, the one or more charged amino residues may be introduced into an active site of the parent polypeptide. In each of the forgoing examples, the one or more charged amino residues may be introduced into a region outside of an active site of the parent polypeptide. In each of the forgoing examples, the conditionally active polypeptide may be a conditionally active antibody and

the one or more charged amino acid residues may be introduced into at least one of complementarity determining regions of the conditionally active antibody.

[22] In each of the forgoing examples, the one or more charged amino acid residues may be less than five charged amino acid residues, or less than four charged amino acid residues, or less than three charged amino acid residues, or less than two charged amino acid residues. In each of the forgoing examples, the one or more charged amino acid residues may be selected from Aspartic acid and Glutamic acid.

[23] In each of the forgoing examples, the conditionally active polypeptide may be reversibly inactivated at the first value of the condition.

[24] In each of the forgoing examples, the first and second assays may be conducted in the presence of a species selected from histidine, histamine, hydrogenated adenosine diphosphate, hydrogen sulfide, hydrogenate adenosine triphosphate, citrate, acetate, lactate, bicarbonate, bisulfide, ammonium, dihydrogen phosphate and combinations thereof.

[25] In each of the forgoing examples, the first value of the condition may be a value of a normal physiological condition that is within a normal range of the physiological condition at a site of administration of the conditionally active polypeptide to a subject, or at a tissue or organ at a site of action of the conditionally active polypeptide of a subject, and the second value of the condition may be a value of an aberrant condition that deviates from the normal range of the physiological condition at the site of administration of the conditionally active polypeptide, or at the tissue or organ at the site of action of the conditionally active polypeptide.

[26] In each of the forgoing examples, the conditionally active polypeptide may be a protein or protein fragment. In each of the forgoing examples, the conditionally active polypeptide may be an antibody, a single chain antibody, and an antibody fragment and the activity is a binding activity to an antigen, an Fc region of an antibody, or an enzyme and the activity is an enzymatic activity, or a receptor, a regulatory protein, a soluble protein, a cytokine and a fragment of a receptor, a regulatory protein, a stress protein, a vault-related protein, a neuron protein, a digestive tract protein, a growth factor, a mitochondrial protein, a cytosolic protein, an animal protein, a structural protein, and a plant protein.

[27] Disclosed is a pharmaceutical composition comprising an effective amount of the conditionally active polypeptide of any one of the forgoing examples and a pharmaceutically acceptable carrier.

[28] In each of the forgoing examples, the pharmaceutical composition may further comprise at least one anti-cancer agent. In each of the forgoing examples, the at least one anti-cancer agent may be a chemotherapeutic agent, a radiation therapeutic agent, a hormonal therapeutic agent, a toxin or an immunotherapeutic agent. In each of the forgoing examples, the at least one anti-cancer agent may be a toxin selected from a taxane, a maytansinoid, an auristatin, a calicheamicin, a duocramycin, a mmae, a soporific, a duomycin, an anthracycline, a CC-1065 analog, docetaxel, a cathepsin, ricin, gelonin, Pseudomonas exotoxin, diphtheria toxin, RNase, and a toxic radioisotope. In each of the forgoing

examples the conditionally active polypeptide may be conjugated to the at least one anti-cancer agent, or at least four anti-cancer agent, or at least six anti-cancer agents.

[29] Disclosed is a use of the conditionally active polypeptide of each of the forgoing examples for treatment of solid tumors, inflamed joints, or brain diseases or disorders, or removal of senescent cells from a subject.

[30] Disclosed is a method of treatment of solid tumors, inflamed joints, or brain diseases or disorders comprising a step of administering a conditionally active polypeptide of each of the forgoing examples.

[31] Disclosed is a nucleic acid molecule that encodes the conditionally active polypeptide of each of forgoing examples. Disclosed is an expression vector comprising the nucleic acid molecule of the forgoing examples or a host cell comprising such an expression vector.

[32] Disclosed is a method for diagnosing the presence of cancer in a subject, comprising steps of (a) contacting cells or tissue of the subject with a detectably labeled conditionally active polypeptide of each of the forgoing examples, wherein the conditionally active polypeptide binds to a target on cancer cells; (b) determining whether the conditionally active polypeptide binds to the cell or tissue of the subject.

[33] In each of the forgoing examples, the contacting of the cells or tissue with the conditionally active polypeptide may occur *in vitro*. In each of the forgoing examples, the contacting of the cells or tissue with the conditionally active polypeptide may occur within said subject.

[34] Disclosed is a bispecific antibody comprising at least one binding domain that contains at least one of the conditionally active polypeptides of the forgoing examples. Disclosed is a chimeric protein containing two different binding or catalytic domains, wherein one or both of the domains comprise at least one of the conditionally active polypeptides of the forgoing examples. Disclosed is an oncolytic virus comprising at least one of the conditionally active polypeptides of the forgoing examples.

[35] Disclosed is a method of preparing a conditionally active polypeptide from a parent polypeptide. In the method, a DNA encoding the parent polypeptide is evolved to increase the net charge of the polypeptide using one or more techniques selected from increasing a total number of codons of charged amino acid residues in the DNA, decreasing a total number of codons of uncharged amino acid residues in the DNA or a combination thereof to create mutant DNAs. The mutant DNAs are expressed to obtain mutant polypeptides; and the conditionally active polypeptide is selected from the mutant polypeptides. The selected conditionally active polypeptide exhibits a decrease in activity in a first assay at a first value of a condition compared to the same activity in a second assay at a second value of the same condition. The charged amino acid residues are selected from Aspartic acid, Glutamic acid, Arginine, Lysine, and Histidine.

[35a] In one aspect, the present disclosure refers to a method of preparing a conditionally active antibody or antibody fragment from a parent antibody, single chain antibody or antibody fragment, said method comprising steps of: i. evolving a DNA encoding the parent antibody, single chain antibody or antibody fragment to increase the net charge of the parent antibody, single chain antibody or antibody fragment using one or more techniques selected from increasing a total number of codons of charged amino acid residues in the DNA, decreasing a total number of codons of uncharged amino acid residues in the DNA and a combination thereof, to create mutant DNAs; ii. expressing the mutant DNAs to obtain mutant antibodies or antibody fragments; and iii. selecting the conditionally active antibody or antibody fragment from the mutant antibodies or antibody fragments which is reversibly inactivated in a first assay at a first value of a condition and exhibits a decrease in activity in the first assay at the first value of the condition compared to the same activity in a second assay at a second value of the same condition and the charged amino acid residues are selected from Aspartic acid, Glutamic acid, Arginine, Lysine, and Histidine.

[36] In each of the foregoing examples, the evolving step may employ a site directed mutagenesis technique, optionally selected from oligonucleotide-mediated mutagenesis, PCR mutagenesis, and cassette mutagenesis. In each of the foregoing examples, the evolving step may employ codon substitution or codon insertion.

[37] In each of the foregoing examples, the charged amino acid residues may be selected from Aspartic acid and Glutamic acid.

[38] In each of the foregoing examples, the one or more codons may be introduced into a region of the DNA that encodes an active site of the parent polypeptide. In each of the foregoing examples, the parent polypeptide may be a parent antibody and the active site may be a complementarity determining region of the parent antibody.

[39] In each of the foregoing examples, the the one or more charged amino residues may be introduced into a region outside of an active site of the parent polypeptide.

[40] In each of the foregoing examples, the region outside of the active site may be a constant region of an antibody, or a framework region of the antibody.

[41] In each of the foregoing examples, the conditionally active polypeptide may be reversibly inactivated under the first value of the condition.

[42] In each of the foregoing examples, the condition may be selected from pH, temperature, osmotic pressure, osmolality, oxidative stress, electrolyte concentration, and concentration of a protein.

[43] In each of the foregoing examples, the first and second assays may be performed in assay solutions comprising a protein found in blood. In each of the foregoing examples, the blood protein may be albumin.

[44] In each of the foregoing examples, the first and second assays may be performed in assay solutions in absence of serum.

[45] In each of the foregoing examples, the value of the second condition may be a value of a normal physiological condition that is within a normal range of the physiological condition at a site of administration of the conditionally active polypeptide to a subject, or at a tissue or organ at a site of action of the conditionally active polypeptide of a subject, and the value of the first condition may be a value of an aberrant condition that deviates from the normal range of the physiological condition at the site of administration of the conditionally active polypeptide, or at the tissue or organ at the site of action of the conditionally active polypeptide.

[46] In each of the foregoing examples, the condition may be pH and the first and second assays may be performed in assay media including at least one species having a molecular weight of less than 900 a.m.u. and a pKa up to 3 units away from the first value of pH.

[47] In each of the foregoing examples, the condition may be pH and the first and second assays may be performed in assay media including at least one species having a molecular weight of less than 900 a.m.u. and a pKa between the second value of pH and the first value of pH.

[48] In each of the foregoing examples, the first and second assays may be performed in assay media including a species selected from histidine, histamine, hydrogenated adenosine diphosphate, hydrogenate adenosine triphosphate, citrate, acetate, lactate, hydrogen sulfide, bisulfide, ammonium, bicarbonate, dihydrogen phosphate and a combination thereof.

[49] In each of the foregoing examples, the selecting step may further comprise selecting the conditionally active polypeptide based on a property selected from affinity, expression level, and humanization.

[50] In each of the foregoing examples, the expression step may employ phage display or a eukaryotic cell production host. In each of the foregoing examples, the expression step may be performed in the eukaryotic cell production host and the selected conditionally active polypeptide may be expressed in the same eukaryotic cell production host.

BRIEF DESCRIPTION OF THE DRAWING

[51] FIG. 1 shows the selectivity's at pH 6.0 over pH 7.4 of the conditionally active antibodies produced in Example 9.

[52] FIG. 2 shows the binding activities assayed in different buffer solutions of several different conditionally active antibodies to an antigen.

[53] FIG. 3 shows the effects of changing the composition of Krebs buffer on the binding activity of a conditionally active antibody.

[54] FIG. 4 shows that the binding activities of three different conditionally active antibodies were dependent on the presence and concentration of bicarbonate at pH 7.4, as described in Example 12.

[55] FIG. 5 is a diagram showing the structure of a chimeric antigen receptor (CAR).

[56] FIG. 6 is a diagram showing the formation of salt bridges in deoxyhemoglobin, where three amino acid residues form two salt bridges that stabilize the T quaternary structure of the deoxyhemoglobin, leading to lower affinity to oxygen.

[57] FIG. 7 shows the activities of conditionally active antibodies against ROR2 in different buffer solutions.

[58] FIG. 8 shows the activities of conditionally active antibodies against Axl in different buffer solutions.

[59] FIGS. 9A-9E show results of a cell killing of A549 cells using a wild-type anti-Axl antibody and conditionally active anti-Axl antibodies at pH 6.0 and pH 7.4 at different antibody concentrations.

[60] FIGS. 10A-10D show binding affinity to human Axl and cynomolgus Axl of conditionally active anti-Axl antibodies in different buffers and at different pH levels.

[61] FIGS. 11A-11H show cell killing of different cell lines at different pH levels by conditionally active anti-Axl antibodies conjugated to duomycin.

WHAT IS CLAIMED IS:

1. A method of preparing a conditionally active antibody or antibody fragment from a parent antibody, single chain antibody or antibody fragment, said method comprising steps of:

i. evolving a DNA encoding the parent antibody, single chain antibody or antibody fragment to increase the net charge of the parent antibody, single chain antibody or antibody fragment using one or more techniques selected from increasing a total number of codons of charged amino acid residues in the DNA, decreasing a total number of codons of uncharged amino acid residues in the DNA and a combination thereof, to create mutant DNAs;

ii. expressing the mutant DNAs to obtain mutant antibodies or antibody fragments; and

iii. selecting the conditionally active antibody or antibody fragment from the mutant antibodies or antibody fragments which is reversibly inactivated in a first assay at a first value of a condition and exhibits a decrease in activity in the first assay at the first value of the condition compared to the same activity in a second assay at a second value of the same condition and the charged amino acid residues are selected from Aspartic acid, Glutamic acid, Arginine, Lysine, and Histidine.

2. The method of claim 1, wherein the evolving step employs site directed mutagenesis selected from oligonucleotide-mediated mutagenesis, PCR mutagenesis, and cassette mutagenesis.

3. The method of any one of claims 1-2, wherein the evolving step comprising introducing one or more codons of charged amino acid residues into the DNA.

4. The method of any one of claims 1-3, wherein the charged amino acid residues are selected from Aspartic acid and Glutamic acid.

5. The method of any one of claims 3-4, wherein the one or more codons are introduced into a region of the DNA that encodes an active site of the parent antibody, single chain antibody or antibody fragment.

6. The method of claim 5, wherein the active site is a complementarity determining region of the parent antibody, single chain antibody or antibody fragment.

7. The method of any one of claims 3-4, wherein the one or more charged amino residues are introduced into a region outside of an active site of the parent antibody, single chain antibody or antibody fragment.

8. The method of claim 7, wherein the region outside of the active site is a constant region of the antibody, single chain antibody or antibody fragment, or a framework region of the antibody, single chain antibody or antibody fragment.

9. The method of any one of claims 1-8, wherein the condition is selected from pH, temperature, osmotic pressure, osmolality, oxidative stress, electrolyte concentration, and concentration of a protein.

10. The method of any one of claims 1-9, wherein the first and second assays are performed in assay solutions comprising a protein found in blood.

11. The method of claim 10, wherein the blood protein is albumin.

12. The method of any one of claims 1-11, wherein the first and second assays are performed in assay solutions in the absence of serum.

13. The method of any one of claims 1-12, wherein the value of the first condition is a value of a normal physiological condition that is within a normal range of the physiological condition at a site of administration of the conditionally active antibody or antibody fragment to a subject, or at a tissue or organ at a site of action of the conditionally active antibody or antibody fragment of a subject, and the value of the second condition is a value of an aberrant condition that deviates from the normal range of the physiological condition at the site of administration of the conditionally active antibody or antibody fragment, or at the tissue or organ at the site of action of the conditionally active antibody or antibody fragment.

14. The method of any one of claims 1-13, wherein the activity is a binding activity to an antigen.

15. The method of any one of claims 1-14, wherein the selecting step further comprises selecting the conditionally active antibody or antibody fragment based on a property selected from affinity, expression level, and humanization,

16. The method of any one of claims 1-15, where the expression step employs phage display or a eukaryotic cell production host.

17. The method of any one of claims 1-3 and 5-16, wherein the charged amino acid residues selected from Aspartic acid, Glutamic acid, Arginine and Lysine.