METHODS OF DECREASING TRISULFIDE BONDS DURING RECOMBINANT PRODUCTION OF POLYPEPTIDES

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit of U.S. Provisional Application Serial No. 62/334,433, filed May 10, 2016, which is incorporated herein by reference in its entirety.

SUBMISSION OF SEQUENCE LISTING ON ASCII TEXT FILE

[0002] The content of the following submission on ASCII text file is incorporated herein by reference in its entirety: a computer readable form (CRF) of the Sequence Listing (file name: 146392036540SEQLIST.txt, date recorded: May 8, 2017, size: 34.1 KB).

FIELD OF THE INVENTION

[0003] The present disclosure relates to cell culture media and methods for decreasing trisulfide bonds in polypeptides produced recombinantly.

BACKGROUND OF THE INVENTION

[0004] Trisulfide bonds are generated by the insertion of an additional sulfur atom into a disulfide bond, thereby resulting in the covalent bonding of three consecutive sulfur atoms. Trisulfide bond formation is a source of heterogeneity in recombinantly produced therapeutic polypeptides. Such heterogeneity is undesirable, as therapeutic products must undergo extensive characterization and meet acceptable standards that ensure product quality and consistency. As such, a demand exists for methods for decreasing the levels of trisulfide bonds during the manufacture of therapeutic polypeptides. There is also a need in the art for minimizing the variability of trisulfide bond levels in therapeutic polypeptides during manufacture. The present disclosure is directed to this and other needs.

BRIEF SUMMARY ~~OF THE INVENTION~~

[0005] Provided is a method for decreasing trisulfide bond levels in a polypeptide comprising: (a) contacting a host cell comprising a nucleic acid encoding the polypeptide with a basal medium, wherein the basal medium comprises one or more of the following components: i) between about 2 μM to about 35μM iron, ii) between about 0.11 μM to about 2 μM riboflavin (vitamin B2), iii) between about 4.5 μM to about 80 μM pyridoxine or

pyridoxal (vitamin B6),iv) between about 3.4 µM to about 23 µM folic acid (vitamin B9), v) between about 0.2 µM to about 2.5 µM cyanocobalamin (vitamin B12), vi) between about 9 mM and about 10 mM hypotaurine; and vii) between about 0 and about 1.58 mM methionine; (b) culturing the host cell to produce the polypeptide; and (c) harvesting the polypeptide produced by the host cell. In one aspect, the disclosure provides a method for decreasing trisulfide bond levels in a polypeptide comprising: (a) contacting a Chinese Hamster Ovary (CHO) cell comprising a nucleic acid encoding the polypeptide with a basal medium, wherein the basal medium comprises: i) between 2 µM to 35 µM iron, ii) between 0.11 µM to 2 µM riboflavin (vitamin B2), iii) between 4.5 µM to 80 µM pyridoxine or pyridoxal (vitamin B6), iv) between 3.4 µM to 23 µM folic acid (vitamin B9), and v) between 0.2 µM to 2.5 µM cyanocobalamin (vitamin B12), or the basal medium comprises: A) between 9 mM and 10 mM hypotaurine; or the basal medium comprises: 1) between 0 and 1.58 mM methionine; (b) culturing the CHO cell to produce the polypeptide; and (c) harvesting the polypeptide produced by the CHO cell. In a related ~~aspect~~example, provided is a method for producing a polypeptide, comprising contacting a host cell comprising a nucleic acid encoding the polypeptide with a basal medium, wherein the basal medium comprises one or more of the following components: between about 2 µM to about 35µM iron, between about 0.11 µM to about 2 µM riboflavin (vitamin B2), between about 4.5 µM to about 80 µM pyridoxine or pyridoxal (vitamin B6), between about 3.4 µM to about 23 µM folic acid (vitamin B9), between about 0.2 µM to about 2.5 µM cyanocobalamin (vitamin B12), between about 9 mM and about 10 mM hypotaurine; and between about 0 and about 1.58 mM methionine; (b) culturing the host cell to produce the polypeptide; and (c)harvesting the polypeptide produced by the host cell. In another aspect, the disclosure provides a method for producing a polypeptide, comprising: (a) contacting a Chinese Hamster Ovary (CHO) cell comprising a nucleic acid encoding the polypeptide with a basal medium, wherein the basal medium comprises: i) between 2 µM to 35 µM iron, ii) between 0.11 µM to 2 µM riboflavin (vitamin B2), iii) between 4.5 µM to 80 µM pyridoxine or pyridoxal (vitamin B6), iv) between 3.4 µM to 23 µM folic acid (vitamin B9), and v) between 0.2 µM to 2.5 µM cyanocobalamin (vitamin B12), or the basal medium comprises: A) between 9 mM and 10 mM hypotaurine; or the basal medium comprises: 1) between 0 and 1.58 mM methionine; (b) culturing the CHO cell to produce the polypeptide; and (c) harvesting the polypeptide produced by the CHO cell.

[0006] In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the harvested polypeptide has a trisulfide bond level less than a polypeptide produced under identical conditions, except that the concentration of the one or more components differs from the concentration specified in (a). In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the basal medium lacks cystine. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the basal medium comprises between about 1.4 mM to 3 mM cysteine or cystine. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the basal medium comprises between about 0 mM to about 1.58 mM methionine and between about 0 mM to about 3 mM cysteine. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the basal medium comprises about 6mM cysteine.

[0007] Also provided is a method for decreasing trisulfide bond levels in a polypeptide comprising: (a) culturing a host cell comprising a nucleic acid encoding the polypeptide in a cell culture medium, wherein the cell culture medium comprises one or more of the following components: i) between about 2 µM to about 35µM iron, ii) between about 0.11 µM to about 2 µM riboflavin (vitamin B2), iii) between about 4.5 µM to about 80 µM pyridoxine or pyridoxal (vitamin B6), iv) between about 3.4 µM to about 23 µM folate/folic acid (vitamin B9), v) between about 0.2 µM to about 2.5 µM cyanocobalamin (vitamin B12),

vi) between about 9 mM and about 10 mM hypotaurine; and vii) between about 0 and about 4.5 mM methionine; (b) producing the polypeptide; (c) and harvesting the polypeptide produced by the host cell. In one aspect, the disclosure provides a method for decreasing trisulfide bond levels in a polypeptide comprising: (a) culturing a Chinese Hamster Ovary (CHO) cell comprising a nucleic acid encoding the polypeptide in a cell culture medium, wherein the cell culture medium comprises: i) between 2 µM to 35 µM iron, ii) between 0.11 µM to 2 µM riboflavin (vitamin B2), iii) between 4.5 µM to 80 µM pyridoxine or pyridoxal (vitamin B6), iv) between 3.4 µM to 23 µM folate/folic acid (vitamin B9), and v) between 0.2 µM to 2.5 µM cyanocobalamin (vitamin B12), or the cell culture medium comprises: A) between 9 mM and 10 mM hypotaurine; or the cell culture medium comprises: 1) between 0 and 2.25 mM methionine; (b) producing the polypeptide; (c) and harvesting the polypeptide produced by the CHO cell. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the concentration of one or more of the components in the cell culture medium is the cumulative concentration of one or more additions after inoculation.

[0008] Provided is a method for decreasing trisulfide bond levels in a polypeptide selected from the group consisting of: a CEA-IL2v immunocytokine, a FAP-IL2v immunocytokine, an anti-CEA/anti-CD3 bispecific antibody, an anti-VEGF/anti-angiopoietin bispecific antibody, an anti-Ang2/anti-VEGF bispecific antibody, an anti-C5 antibody, and an anti-CD40 antibody , the method comprising: (a) culturing a host cell comprising a nucleic acid encoding the polypeptide in a cell culture medium, wherein the cell culture medium comprises one or more of the following components: i) between about 2 µM to about 35µM iron, ii) between about 0.11 µM to about 2 µM riboflavin (vitamin B2), iii) between about 4.5 µM to about 80 µM pyridoxine or pyridoxal (vitamin B6), iv) between about 3.4 µM to about 23 µM folate/folic acid (vitamin B9), v) between about 0.2 µM to about 2.5 µM cyanocobalamin (vitamin B12), vi) between about 9 mM and about 10 mM hypotaurine; and vii) between about 0 and about 4.5 mM methionine; (b) producing the polypeptide; (c) and harvesting the polypeptide produced by the host cell. In one aspect, the disclosure provides a method for decreasing trisulfide bond levels in a polypeptide selected from the group consisting of: a CEA-IL2v immunocytokine, a FAP-IL2v immunocytokine, an anti-CEA/anti-CD3 bispecific antibody, an anti-VEGF/anti-angiopoietin bispecific antibody, an anti-Ang2/anti-VEGF bispecific antibody, an anti-C5 antibody, and an anti-CD40 antibody, the method comprising: (a) culturing a Chinese Hamster Ovary (CHO) cell comprising a nucleic acid encoding the polypeptide in a cell culture medium, wherein the cell culture medium comprises: i) between 2 µM to 35 µM iron, ii) between 0.11 µM to 2 µM riboflavin (vitamin B2), iii) between 4.5 µM to 80 µM pyridoxine or pyridoxal (vitamin B6), iv) between 3.4 µM to 23 µM folate/folic acid (vitamin B9), v) between 0.2 µM to 2.5 µM cyanocobalamin (vitamin B12), or the cell culture medium comprises: A) between 9 mM and 10 mM hypotaurine; or the cell culture medium comprises: 1) between 0 and 2.25 mM methionine; (b) producing the polypeptide; (c) and harvesting the polypeptide produced by the CHO cell.

[0009] Also provided is a method for decreasing trisulfide bond levels in a polypeptide selected from the group consisting of: a CEA-IL2v immunocytokine, a FAP-IL2v immunocytokine, an anti-CEA/anti-CD3 bispecific antibody, an anti-VEGF/anti-angiopoietin bispecific antibody, an anti-Ang2/anti-VEGF bispecific antibody, an anti-C5 antibody and an anti-CD40 antibody , the method comprising: (a) culturing a host cell comprising a nucleic acid encoding the polypeptide in a cell culture medium, wherein the cell culture medium comprises one or more of the following components: i) between about 2 µM to about 35µM iron, and ii) between about 0 and about 4.5 mM methionine; (b) producing the polypeptide; and (c) harvesting the polypeptide produced by the host cell. In one aspect, the disclosure provides a method for decreasing trisulfide bond levels in a polypeptide selected from the group consisting of: a CEA-IL2v immunocytokine, a FAP-IL2v immunocytokine, an anti-CEA/anti-CD3 bispecific antibody, an anti-VEGF/anti-angiopoietin bispecific antibody, an anti-Ang2/anti-VEGF bispecific antibody, an anti-C5 antibody, and an anti-CD40 antibody, the method comprising: (a) culturing a Chinese Hamster Ovary (CHO) cell comprising a nucleic acid encoding the polypeptide in a cell culture medium, wherein the cell culture medium comprises one or more of the following components: i) between 2 µM to 35 µM iron, and ii) between 0 and 2.25 mM methionine; (b) producing the polypeptide; and (c) harvesting the polypeptide produced by the CHO cell.

[0010] In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the method further comprises at least one feed, and wherein the feed medium lacks one or more of the following: iron, riboflavin, pyridoxine, pyridoxal, folic acid, and cyanocobalamin. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the feed is a batch feed. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the batch feed medium lacks cystine. In certain

~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the batch feed medium lacks cysteine. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the batch feed medium lacks methionine. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the iron is ferric iron (Fe^{3+}) or ferrous iron (Fe^{2+}).

[0011] In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the method further comprises: (I) supplementing the culture of said host cell with a chelating agent and a reducing agent prior to harvest; (II) supplementing a pre-harvest cell culture fluid (PHCCF) of said host cell with a chelating agent and a reducing agent; or (III) supplementing a harvested cell culture fluid (HCCF) of said host cell with a chelating agent and a reducing agent following harvest.

[0012] Also provided is a method for decreasing level of trisulfide bonds in a polypeptide produced by a host cell comprising: (i) supplementing a culture of said host cell with a reducing agent and a chelating agent prior to harvest; (ii) supplementing a pre-harvest cell culture fluid (PHCCF) of said host cell with a chelating agent and a reducing agent; or (iii) supplementing a harvested cell culture fluid (HCCF) of said host cell with a reducing agent and a chelating agent.

[0013] In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the culture, the PHCCF, or the HCCF of said host cell is supplemented with the chelating agent prior to being supplemented with the reducing agent. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the PHCCF, or the HCCF of said host cell is supplemented with the chelating agent between about 60 minutes to about 30 minutes prior to being supplemented with the reducing agent. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the chelating agent and the reducing agent are maintained in the culture, the PHCCF, or the HCCF of said host cell for about 30 minutes to about 4 days. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the culture, the PHCCF, or the HCCF of said host cell is maintained at a temperature between about 15°C and about 37°C. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the culture, the PHCCF, or the HCCF of said host cell is maintained pH between about 6.5 to about 7.5. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the amount of dissolved oxygen (DO) in the culture, the PHCCF, or the HCCF of said host cell is at least about 15%. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples

above, the culture, the PHCCF, or the HCCF of said host cell is maintained at a temperature between about 15°C and about 37°C and at a pH between about 6.5 to about 7.5, and wherein the amount of dissolved oxygen (DO) in the culture or HCCF of said host cell is at least about 15%.

[0014] In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the reducing agent is selected from the group consisting of: glutathione (GSH), L-glutathione (L-GSH), cysteine, L-cysteine, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 2,3-tert-butyl-4-hydroxyanisole, 2,6-di-tert-butyl-4-methylphenol, 3-aminopropane-1-sulfonic acid, adenosylhomocysteine, anserine, B-alanine, B-carotene, butylated hydroxyanisole, butylated hydroxytoluene, carnosine, carvedilol, curcumin, cysteamine, cysteamine hydrochloride, dexamethasone, diallyldisulfide, DL-lanthionine, DL-thiorphan, ethoxyquin, gallic acid, gentisic acid sodium salt hydrate, glutathione disulfide, glutathione reduced ethyl ester, glycine, hydrocortisone, hypotaurine, isethionic acid ammonium salt, L-cysteine-glutathione Disulfide, L-cysteinesulfinic acid monohydrate, lipoic acid, reduced lipoic acid, mercaptopropionyl glycine, methionine, methylenebis(3-thiopropionic acid), oxalic acid, quercitrin hydrate, resveratrol, retinoic acid, S-carboxymethyl-L-cysteine, selenium, selenomethionine, silver diethyldithiocarbamate, taurine, thiolactic acid, tricine, vitamin C, vitamin E, vitamin B1, vitamin B2, vitamin B3, vitamin B4, vitamin B5, vitamin B6, and vitamin B11. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the reducing agent is selected from the group consisting of: cysteine and L-cysteine. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the reducing agent is L-cysteine, and wherein the L-cysteine is added to the culture or HCCF of said host cell to achieve a final concentration between about 3 mM and about 6 mM.

[0015] In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the chelating agent is selected from the group consisting of: ethylenediaminetetraacetic acid (EDTA), nitrilotriacetic acid (NTA), ethylenediamine-N,N'-disuccinic acid (EDDS), citrate, oxalate, tartrate, ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA), diethylenetriaminepentaacetic acid (DTPA), 5-sulfosalicylic acid, N,N-dimethyldodecylamine N-oxide, dithiooxamide, ethylenediamine, salicylaldoxime, N-(2'-hydroxyethyl)iminodiacetic acid (HIMDA), oxine quinolinol, and sulphoxine. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the chelating agent is selected from the group consisting of: ethylenediaminetetraacetic acid (EDTA),

nitrilotriacetic acid (NTA), ethylenediamine-N,N'-disuccinic acid (EDDS), and citrate. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the chelating agent is added to the culture or the HCCF of said host cell to achieve a final concentration of 20mM.

[0016] In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the polypeptide is secreted into the cell culture medium. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the method further comprises a step of purifying the harvested polypeptide. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the host cell is a recombinant host cell. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the host cell is a mammalian cell. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the mammalian cell is a CHO cell. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the method further comprises measuring the level of trisulfide bonds in the polypeptide. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the average % trisulfide bonds in the polypeptide is less than about 20%, less than about 10% less than about 5%, less than about 1%, less than about 0.5%, or less than about 0.1%.

[0017] In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the polypeptide is an antibody or fragment thereof. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the polypeptide is an antibody fragment, and wherein the antibody fragment is selected from the group consisting of: a Fab, a Fab', an $F(ab')_2$, an scFv, an $(scFv)_2$, a dAb, a complementarity determining region (CDR) fragment, a linear antibody, a single-chain antibody molecule, a minibody , a diabody, and multispecific antibody formed from antibody fragments. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the antibody or fragment thereof binds to an antigen selected from the group consisting of: BMPR1B, E16, STEAP1, 0772P, MPF, Napi3b, Sema 5b, PSCA hlg, ETBR, MSG783, STEAP2, TrpM4, C5, CRIPTO, CD21, CD79b, FcRH2, HER2, NCA, MDP, IL20Rα, Brevican, EphB2R, ASLG659, PSCA, GEDA, BAFF-R, CD22, CD79a, CXCR5, HLA-DOB, P2X5, CD72, LY64, FcRH1, IRTA2, TENB2, PMEL17, TMEFF1, GDNF-Ra1, Ly6E, TMEM46, Ly6G6D, LGR5, RET, LY6K, GPR19, GPR54, ASPHD1, Tyrosinase, TMEM118, GPR172A, CD33, CLL-1, OX40, α4β7 and αEβ7 integrin heterodimers, IL-13, CD-20, FGFR, influenza A, influenza B, amyloid beta, HER3, complement factor D, IL-22c, PD-L1, PD-L2, PD-1, VEGF, Angiopoietin 2, CD3, FAP,

CEA, and IL-6. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the polypeptide is an antibody, and wherein the antibody is a bispecific antibody. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the bispecific antibody is an anti-VEGF/anti-angiopoietin bispecific antibody, an anti-CEA/anti-CD3 bispecific antibody, or an anti-Ang2/anti-VEGF bispecific antibody. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the polypeptide is an immunocytokine. In certain ~~embodiment~~examples according to (or as applied to) any of the ~~embodiment~~examples above, the immunocytokine is CEA-IL2v or FAP-IL2v.

[0018] Also provided is the use of between about 0 and about 4.5 μM methionine in a cell culture medium for decreasing trisulfide bond levels in a polypeptide selected from the group consisting of: a CEA-IL2v immuocytokine, a FAP-IL2v immunocytokine, an anti-CEA/anti-CD3 bispecific antibody, an anti-VEGF/anti-angiopoietin bispecific antibody, an anti-Ang2/anti-VEGF bispecific antibody, an anti-C5 antibody, and an anti-CD40 antibody. In one aspect, the disclosure provides use of between 0 and 2.25 mM methionine in a cell culture medium for decreasing trisulfide bond levels in a polypeptide produced by a CHO cell cultured in said cell culture medium, wherein the polypeptide is selected from the group consisting of: a CEA-IL2v immuocytokine, a FAP-IL2v immunocytokine, an anti-CEA/anti-CD3 bispecific antibody, an anti-VEGF/anti-angiopoietin bispecific antibody, an anti-Ang2/anti-VEGF bispecific antibody, an anti-C5 antibody, and an anti-CD40 antibody. In another aspect, the disclosure provides a method of manufacturing a medicament comprising the method as disclosed herein.

[0019] It is to be understood that one, some, or all of the properties of the various ~~embodiment~~examples described herein may be combined to form other ~~embodiment~~examples of the present ~~invention~~disclosure. These and other ~~aspect~~examples of the ~~invention~~disclosure will become apparent to one of skill in the art.

[0020] All publications, patents, and patent applications cited herein are incorporated by reference in their entirety for all purposes.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] **FIG. 1** provides the results of experiments performed to assess the % trisulfide bond in anti-FluB incubated in a cell-free system containing Medium 1 + cysteine (Cys); Medium 1 + Cys + Fe (iron); Medium 1 + cystine (Cys-Cys); Medium 1 + Cys-Cys + Fe; Medium 2 + Cys; Medium 2 + Cys + Fe; Medium 2 + Cys-Cys; or Medium 1 + Cys-Cys + Fe.

[0022] **FIG. 2A** provides the results of experiments performed to assess the % trisulfide bond in anti-FluB incubated in Medium 1 that was supplemented with one or more of the following components: (a) Cys-Cys, (b) Fe, and (c) B vitamins (riboflavin, pyridoxine, folic acid, and cyanocobalamin).

[0023] **FIG. 2B** provides the results of experiments performed to assess the % trisulfide bond in an anti-OX40 antibody incubated in Medium 1 that was supplemented with

CLAIMS

What is claimed is:

1. A method for decreasing trisulfide bond levels in a polypeptide comprising:

(a) contacting a Chinese Hamster Ovary (CHO) ~~host~~ cell comprising a nucleic acid encoding the polypeptide with a basal medium, wherein the basal medium comprises ~~one or more of the following components~~:

i) between ~~about~~ 2 μM to ~~about~~ 35 μM iron,

ii) between ~~about~~ 0.11 μM to ~~about~~ 2 μM riboflavin (vitamin B2),

iii) between ~~about~~ 4.5 μM to ~~about~~ 80 μM pyridoxine or pyridoxal (vitamin B6),

iv) between ~~about~~ 3.4 μM to ~~about~~ 23 μM folic acid (vitamin B9), and

v) between ~~about~~ 0.2 μM to ~~about~~ 2.5 μM cyanocobalamin (vitamin B12),

or the basal medium comprises:

~~vi~~A) between ~~about~~ 9 mM and ~~about~~ 10 mM hypotaurine; ~~and~~

or the basal medium comprises:

~~vii~~1) between ~~about~~ 0 and ~~about~~ 1.58 mM methionine;

(b) culturing the CHO ~~host~~ cell to produce the polypeptide; and

(c) harvesting the polypeptide produced by the CHO ~~host~~ cell.

2. A method for producing a polypeptide, comprising:

(a) contacting a Chinese Hamster Ovary (CHO) ~~host~~ cell comprising a nucleic acid encoding the polypeptide with a basal medium, wherein the basal medium comprises ~~one or more of the following components~~:

i) between ~~about~~ 2 μM to ~~about~~ 35 μM iron,

ii) between ~~about~~ 0.11 μM to ~~about~~ 2 μM riboflavin (vitamin B2),

iii) between ~~about~~ 4.5 μM to ~~about~~ 80 μM pyridoxine or pyridoxal (vitamin B6),

iv) between ~~about~~ 3.4 μM to ~~about~~ 23 μM folic acid (vitamin B9), and

v) between ~~about~~ 0.2 μM to ~~about~~ 2.5 μM cyanocobalamin (vitamin B12),

or the basal medium comprises:

~~vi~~A) between ~~about~~ 9 mM and ~~about~~ 10 mM hypotaurine; ~~and~~

or the basal medium comprises:

~~vii~~1) between ~~about~~ 0 and ~~about~~ 1.58 mM methionine;

(b) culturing the CHO ~~host~~ cell to produce the polypeptide; and

(c) harvesting the polypeptide produced by the CHO ~~host~~ cell.

3. The method of claim 1 or claim 2, whereby the harvested polypeptide has a trisulfide bond level less than a polypeptide produced under identical conditions, except that the concentration of i)-v), the concentration of A) ~~one~~ or the concentration of 1) ~~more components~~ differs from the concentration of i)-v), the concentration of A) or the concentration of 1) specified in (a).

4. The method of any one of claims 1-3, wherein the basal medium lacks cystine.

5. The method of any one of claims 1-3, wherein the basal medium comprises between ~~about~~ 1.4 mM to 3 mM cysteine or cystine.

6. The method of any one of claims 1-4, wherein the basal medium comprises between ~~about~~ 0 mM to ~~about~~ 1.58 mM methionine and between ~~about~~ 0 mM to ~~about~~ 3 mM cysteine.

7. The method of any one of claims 1-3, wherein the basal medium comprises ~~about~~ 6mM cysteine.

8. A method for decreasing trisulfide bond levels in a polypeptide comprising:

(a) culturing a Chinese Hamster Ovary (CHO) ~~host~~ cell comprising a nucleic acid encoding the polypeptide in a cell culture medium, wherein the cell culture medium comprises ~~one or more of the following components~~:

i) between ~~about~~ 2 µM to ~~about~~ 35 µM iron,

ii) between ~~about~~ 0.11 µM to ~~about~~ 2 µM riboflavin (vitamin B2),

iii) between ~~about~~ 4.5 µM to ~~about~~ 80 µM pyridoxine or pyridoxal (vitamin B6),

iv) between ~~about~~ 3.4 µM to ~~about~~ 23 µM folate/folic acid (vitamin B9), and

v) between ~~about~~ 0.2 µM to ~~about~~ 2.5 µM cyanocobalamin (vitamin B12),

or the cell culture medium comprises:

~~vi~~A) between ~~about~~ 9 mM and ~~about~~ 10 mM hypotaurine; ~~and~~

or the cell culture medium comprises:

~~vii~~1) between ~~about~~ 0 and ~~about 4.5~~2.25 mM methionine;

(b) producing the polypeptide;

(c) and harvesting the polypeptide produced by the CHO ~~host~~ cell.

9. The method of claim 8, wherein the concentration of i)-v), the concentration of A) ~~one~~ or the concentration of 1) ~~more of the components~~ in the cell culture medium is the cumulative concentration of one or more additions after inoculation.

10. A method for decreasing trisulfide bond levels in a polypeptide selected from the group consisting of: a CEA-IL2v immunocytokine, a FAP-IL2v immunocytokine, an anti-CEA/anti-CD3 bispecific antibody, an anti-VEGF/anti-angiopoietin bispecific antibody, an anti-Ang2/anti-VEGF bispecific antibody, an anti-C5 antibody, and an anti-CD40 antibody, the method comprising:

(a) culturing a Chinese Hamster Ovary (CHO) ~~host~~ cell comprising a nucleic acid encoding the polypeptide in a cell culture medium, wherein the cell culture medium comprises ~~one or more of the following components~~:

i) between ~~about~~ 2 µM to ~~about~~ 35 µM iron,

ii) between ~~about~~ 0.11 µM to ~~about~~ 2 µM riboflavin (vitamin B2),

iii) between ~~about~~ 4.5 µM to ~~about~~ 80 µM pyridoxine or pyridoxal (vitamin B6),

iv) between ~~about~~ 3.4 µM to ~~about~~ 23 µM folate/folic acid (vitamin B9),

v) between ~~about~~ 0.2 µM to ~~about~~ 2.5 µM cyanocobalamin (vitamin B12),

or the cell culture medium comprises:

~~vi~~A) between ~~about~~ 9 mM and ~~about~~ 10 mM hypotaurine; ~~and~~

or the cell culture medium comprises:

~~vii~~1) between ~~about~~ 0 and ~~about 4.5~~2.25 mM methionine;

(b) producing the polypeptide;

(c) and harvesting the polypeptide produced by the CHO ~~host~~ cell.

11. A method for decreasing trisulfide bond levels in a polypeptide selected from the group consisting of: a CEA-IL2v immunocytokine, a FAP-IL2v immunocytokine, an anti-

CEA/anti-CD3 bispecific antibody, an anti-VEGF/anti-angiopoietin bispecific antibody, an anti-Ang2/anti-VEGF bispecific antibody, an anti-C5 antibody, and an anti-CD40 antibody , the method comprising:

(a) culturing a Chinese Hamster Ovary (CHO) ~~host~~ cell comprising a nucleic acid encoding the polypeptide in a cell culture medium, wherein the cell culture medium comprises one or more of the following components:

i) between ~~about~~ 2 μM to ~~about~~ 35 μM iron, and

ii) between ~~about~~ 0 and ~~about 4.5~~ 2.25 mM methionine;

(b) producing the polypeptide; and

(c) harvesting the polypeptide produced by the CHO ~~host~~ cell.

12. The method of any one of claims 1-11, wherein the method further comprises at least one feed, and wherein the feed medium lacks one or more of the following: iron, riboflavin, pyridoxine, pyridoxal, folic acid, and cyanocobalamin.

13. The method of claim 12, wherein the feed is a batch feed.

14. The method of claim 13, wherein the batch feed medium lacks cystine.

15. The method of claim 13 or 14, wherein the batch feed medium lacks cysteine.

16. The method of any one of claims 13-15, wherein the batch feed medium lacks methionine.

17. The method of any one of claims 1-16, wherein the iron is ferric iron (Fe^{3+}) or ferrous iron (Fe^{2+}).

18. The method of any one of claims 1-17, wherein the method further comprises:

(I) supplementing the culture of said CHO ~~host~~ cell with a chelating agent and a reducing agent prior to harvest;

(II) supplementing a pre-harvest cell culture fluid (PHCCF) of said CHO ~~host~~ cell with a chelating agent and a reducing agent; or

(III) supplementing a harvested cell culture fluid (HCCF) of said CHO ~~host~~ cell with a chelating agent and a reducing agent following harvest.

~~19. A method for decreasing level of trisulfide bonds in a polypeptide produced by a host cell comprising:~~

~~(i) supplementing a culture of said host cell with a reducing agent and a chelating agent prior to harvest;~~

~~(ii) supplementing a pre-harvest cell culture fluid (PHCCF) of said host cell with a chelating agent and a reducing agent; or~~

~~(iii) supplementing a harvested cell culture fluid (HCCF) of said host cell with a reducing agent and a chelating agent.~~

~~20~~19. The method of ~~any one of claims 15-16~~claim 18, wherein the culture, the PHCCF, or the HCCF of said CHO ~~host~~ cell is supplemented with the chelating agent prior to being supplemented with the reducing agent.

~~21~~20. The method of claim ~~20~~19, wherein the culture, the PHCCF, or the HCCF of said CHO ~~host~~ cell is supplemented with the chelating agent between ~~about~~ 60 minutes to ~~about~~ 30 minutes prior to being supplemented with the reducing agent.

~~22~~21. The method of any one of claims 18-~~21~~20, wherein the chelating agent and the reducing agent are maintained in the culture, the PHCCF, or the HCCF of said CHO ~~host~~ cell for ~~about~~ 30 minutes to ~~about~~ 4 days.

~~23~~22. The method of any one of claims 18-~~22~~21, wherein the culture, the PHCCF, or the HCCF of said CHO ~~host~~ cell is maintained at a temperature between ~~about~~ 15°C and ~~about~~ 37°C.

~~24~~23. The method of any one of claims 18-~~23~~22, wherein the culture, the PHCCF, or the HCCF of said CHO~~host~~ cell is maintained pH between ~~about~~ 6.5 to ~~about~~ 7.5.

~~25~~24. The method of any one of claims 18-~~24~~23, wherein the amount of dissolved oxygen (DO) in the culture, the PHCCF, or the HCCF of said CHO ~~host~~ cell is at least ~~about~~ 15%.

2625. The method of any one of claims 18-2221, wherein the culture, the PHCCF, or the HCCF of said CHO host cell is maintained at a temperature between about 15°C and about 37°C and at a pH between about 6.5 to about 7.5, and wherein the amount of dissolved oxygen (DO) in the culture or HCCF of said CHO host cell is at least about 15%.

2726. The method of any one of claims 18-2625, wherein the reducing agent is selected from the group consisting of: glutathione (GSH), L-glutathione (L-GSH), cysteine, L-cysteine, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 2,3-tert-butyl-4-hydroxyanisole, 2,6-di-tert-butyl-4-methylphenol, 3-aminopropane-1-sulfonic acid, adenosylhomocysteine, anserine, B-alanine, B-carotene, butylated hydroxyanisole, butylated hydroxytoluene, carnosine, carvedilol, curcumin, cysteamine, cysteamine hydrochloride, dexamethasone, diallyldisulfide, DL-lanthionine, DL-thiorphan, ethoxyquin, gallic acid, gentisic acid sodium salt hydrate, glutathione disulfide, glutathione reduced ethyl ester, glycine, hydrocortisone, hypotaurine, isethionic acid ammonium salt, L-cysteine-glutathione Disulfide, L-cysteinesulfinic acid monohydrate, lipoic acid, reduced lipoic acid, mercaptopropionyl glycine, methionine, methylenebis(3-thiopropionic acid), oxalic acid, quercitrin hydrate, resveratrol, retinoic acid, S-carboxymethyl-L-cysteine, selenium, selenomethionine, silver diethyldithiocarbamate, taurine, thiolactic acid, tricine, vitamin C, vitamin E, vitamin B1, vitamin B2, vitamin B3, vitamin B4, vitamin B5, vitamin B6, and vitamin B11.

2827. The method of claim 2726, wherein the reducing agent is selected from the group consisting of: cysteine and L-cysteine.

2928. The method of claim 2827, wherein the reducing agent is L-cysteine, and wherein the L-cysteine is added to the culture or HCCF of said CHO host cell to achieve a final concentration between about 3 mM and about 6 mM.

3029. The method of any one of claims 18-2928, wherein the chelating agent is selected from the group consisting of: ethylenediaminetetraacetic acid (EDTA), nitrilotriacetic acid (NTA), ethylenediamine-N,N'-disuccinic acid (EDDS), citrate, oxalate, tartrate, ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA), diethylenetriaminepentaacetic acid (DTPA), 5-sulfosalicylic acid, N,N-dimethyldodecylamine N-oxide, dithiooxamide, ethylenediamine,

salicylaldoxime, N-(2'-hydroxyethyl)iminodiacetic acid (HIMDA), oxine quinolinol, and sulphoxine.

~~31~~30. The method of claim ~~30~~29, wherein the chelating agent is selected from the group consisting of: ethylenediaminetetraacetic acid (EDTA), nitrilotriacetic acid (NTA), ethylenediamine-N,N'-disuccinic acid (EDDS), and citrate.

~~32~~31. The method of claim ~~31~~30, wherein the chelating agent is added to the culture or the HCCF of said CHO ~~host~~ cell to achieve a final concentration of 20mM.

~~33~~32. The method of any one of claims 1-~~32~~31, wherein the polypeptide is secreted into the cell culture medium.

~~34~~33. The method of any one of claims 1-~~33~~32, further comprising a step of purifying the harvested polypeptide.

~~35~~34. The method of any one of claims 1-~~34~~33, wherein the CHO ~~host~~ cell is a recombinant CHO ~~host~~ cell.

~~36. The method of any one of claims 1-35, wherein the host cell is a mammalian cell.~~

~~37. The method of claim 36, wherein the mammalian cell is a CHO cell.~~

~~38~~35. The method of any one of claims 1-~~37~~34, wherein the method further comprises measuring the level of trisulfide bonds in the polypeptide.

~~39~~36. The method of any one of claims 1-~~38~~35, wherein the average % trisulfide bonds in the polypeptide is less than ~~about~~ 20%, less than ~~about~~ 10% less than ~~about~~ 5%, less than ~~about~~ 1%, less than ~~about~~ 0.5%, or less than ~~about~~ 0.1%.

~~40~~37. The method of any one of claims 1-9 and 12-~~39~~36, wherein the polypeptide is an antibody or fragment thereof.

~~41~~38. The method of claim ~~40~~37, wherein the polypeptide is an antibody fragment, and wherein the antibody fragment is selected from the group consisting of: a Fab, a Fab', an $F(ab')_2$, an scFv, an $(scFv)_2$, a dAb, a complementary determining region (CDR) fragment, a linear antibody, a single-chain antibody molecule, a minibody , a diabody, and multispecific antibody formed from antibody fragments.

~~42~~39. The method of claim ~~40~~37, wherein the antibody or fragment thereof binds to an antigen selected from the group consisting of: BMPR1B, E16, STEAP1, 0772P, MPF, Napi3b, Sema 5b, PSCA hlg, ETBR, MSG783, STEAP2, TrpM4, CRIPTO, CD21, CD79b, FcRH2, HER2, NCA, MDP, IL20Rα, Brevican, EphB2R, ASLG659, PSCA, GEDA, BAFF-R, CD22, CD79a, CXCR5, HLA-DOB, P2X5, CD72, LY64, FcRH1, IRTA2, TENB2, PMEL17, TMEFF1, GDNF-Ra1, Ly6E, TMEM46, Ly6G6D, LGR5, RET, LY6K, GPR19, GPR54, ASPHD1, Tyrosinase, TMEM118, GPR172A, CD33, CLL-1, C5, OX40, α4β7 and αEβ7 integrin heterodimers, IL-13, CD-20, FGFR, influenza A, influenza B, amyloid beta, HER3, complement factor D, IL-22c, PD-L1, PD-L2, PD-1, VEGF, Angiopoietin 2, CD3, FAP, CEA, and IL-6.

~~43~~40. The method of claim ~~40~~37, wherein the polypeptide is an antibody, and wherein the antibody is a bispecific antibody.

~~44~~41. The method of claim ~~43~~40, wherein the bispecific antibody is an anti-VEGF/anti-angiopoietin bispecific antibody, an anti-CEA/anti-CD3 bispecific antibody, or an anti-Ang2/anti-VEGF bispecific antibody.

~~45~~42. The method of any one of claims 1-9 and 12-~~39~~36, wherein the polypeptide is an immunocytokine.

~~46~~43. The method of claim ~~45~~42, wherein the immunocytokine is CEA-IL2v or FAP-IL2v.

~~47~~44. Use of between ~~about~~ 0 and ~~about 4.5 µM~~2.25 mM methionine in a cell culture medium for decreasing trisulfide bond levels in a polypeptide produced by a CHO cell cultured in said cell culture medium, wherein the polypeptide is selected from the group consisting of: a CEA-IL2v immuocytokine, a FAP-IL2v immunocytokine, an anti-CEA/anti-

CD3 bispecific antibody, an anti-VEGF/anti-angiopoietin bispecific antibody, an anti-Ang2/anti-VEGF bispecific antibody, an anti-C5 antibody, and an anti-CD40 antibody.

~~48~~45. The method or the use of any one of claims 1-44, wherein the method is used for producing the polypeptide~~A polypeptide produced according to any one of the preceding claims~~.

~~49~~46. The ~~polypeptide~~ method of claim ~~48~~45, wherein the average % trisulfide in the polypeptide is less than ~~about~~ 20%, less than ~~about~~ 10% less than ~~about~~ 5%, less than ~~about~~ 1%, less than ~~about~~ 0.5%, or less than ~~about~~ 0.1%.

47. A method of manufacturing a medicament comprising the method of claim 45 or claim 46.