

EXPRESSION VECTOR SYSTEMS WITH TWO COPIES OF LIGHT CHAIN

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority to U.S. Provisional Patent Application No. 63/623,121, filed January 19, 2024, which is hereby incorporated by reference in its entirety.

FIELD

[0002] The present disclosure provides expression systems for producing multi-specific antibodies having only one Fab or having two Fabs that are identical, host cells comprising such expression systems, including but not limited to Chinese hamster ovary (CHO) cells, and methods for producing multi-specific antibodies utilizing such expression systems.

SUBMISSION OF SEQUENCE LISTING

[0003] The content of the following Sequence Listing XML is incorporated herein by reference in its entirety: file name: 10837-WO01-SEC, date created: January 13, 2025; size: 11,756 bytes.

BACKGROUND

[0004] Due to their broad utility, biologics are used worldwide in a variety of applications, such as therapeutics and diagnostics. Mammalian cell lines are the predominant expression systems for biologics, with Chinese hamster ovary (CHO) cells being the predominant cellular factory. (See Lalonde *et al.*, 2017, J Biotechnol 251:128-140.) Particularly with the advent of biosimilars, speed-to-market and cost-efficiency are now more important than ever before.

[0005] The costs of manufacturing biologics are high due to their complex production strategies, which involve multi-step processes involving the selection of optimal cell lines, culturing production cells in large quantities, and purification of the desired biologic from the cell harvest. Manufacturing is even more complex for new antibody modalities such as multi-specific antibodies having three unique antibody chains. While these costs are decreasing due to improvements in all facets of production, costs can still be prohibitive in their widespread adoption as front-line therapies.

[0006] In order to make biological therapeutics more accessible to patients, decreasing the cost of goods for the manufacturing process is an attractive proposition. One way to achieve this objective is to reduce the cost of goods by increasing the titers associated with production cell lines. The right vector configuration can help to optimize expression levels of different chains in recombinant proteins, particularly for three-chain molecules, resulting in more balanced chain expression, reduced impurities, and higher product quality.

[0007] U.S. Patent No. 11,396,557 describes expression of a bispecific antibody with two different Fabs expressed from two vectors having a common light chain and heavy chains having different heavy chain variable regions.

[0008] However, there still exists a need for expression vector systems, which, when transfected into host cell lines, produce recombinant proteins at high titers, with minimal impact on or potential improvement to product quality attributes, including expression vector systems for recombinant proteins in which the Fab regions are identical or only one Fab is present. Such expression vector systems would benefit the process development of biologics.

SUMMARY

[0009] The present disclosure provides an expression system comprising: 1) a first expression vector which comprises a polynucleotide sequence which comprises the following elements in 5' to 3' order: a) a first promoter operably linked to a nucleotide sequence encoding a light chain followed by a polyadenylation (polyA) signal sequence; b) a second promoter operably linked to a nucleotide sequence encoding a heavy chain, a heavy chain fusion, or a Fc fusion followed by a second polyA signal sequence; and c) a third promoter operably linked to a nucleotide sequence encoding a selectable marker followed by a third polyA signal sequence; and 2) a second expression vector which comprises a polynucleotide sequence which comprises the following elements in 5' to 3' order: a) a first promoter operably linked to a nucleotide sequence encoding an identical copy of the light chain followed by a polyA signal sequence; b) a second promoter operably linked to a nucleotide sequence encoding a heavy chain, a heavy chain fusion, or a Fc fusion followed by a second polyA signal sequence; and c) a third promoter operably linked to a nucleotide sequence encoding a selectable marker followed by a third polyA signal sequence, wherein, when expressed in a host cell, the expression system produces a multi-specific antibody that has only one Fab or has two Fabs that are identical.

[0010] In some embodiments, the multi-specific antibody has only one Fab.

[0011] In some embodiments, the multi-specific antibody has two Fabs that are identical.

[0012] In some embodiments, the multi-specific antibody is a trispecific antibody. For example, in some embodiments, the trispecific antibody has 1) a first heavy chain fusion having a VH or a cytokine fused to either the N-terminus or the C-terminus of the heavy chain portion of the first heavy chain fusion; and 2) a second heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the second heavy chain fusion.

[0013] In some embodiments, the trispecific antibody has 1) a first heavy chain fusion having a VH fused to either the N-terminus or the C-terminus of the heavy chain portion of the first heavy chain fusion; and 2) a second heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the second heavy chain fusion. In some embodiments, the trispecific antibody has 1) a first heavy chain fusion having a VH fused to the N-terminus of the heavy chain portion of the first heavy chain fusion; and 2) a second heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the second heavy chain fusion. In some embodiments, the trispecific antibody has 1) a first heavy chain fusion having a VH fused to the C-terminus of the heavy chain portion of the first

heavy chain fusion; and 2) a second heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the second heavy chain fusion.

[0014] In some embodiments, the trispecific antibody has 1) a first heavy chain fusion having a cytokine fused to either the N-terminus or the C-terminus of the heavy chain portion of the first heavy chain fusion; and 2) a second heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the second heavy chain fusion. In some embodiments, the trispecific antibody has 1) a first heavy chain fusion having a cytokine fused to the N-terminus of the heavy chain portion of the first heavy chain fusion; and 2) a second heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the second heavy chain fusion. In some embodiments, the trispecific antibody has 1) a first heavy chain fusion having a cytokine fused to the C-terminus of the heavy chain portion of the first heavy chain fusion; and 2) a second heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the second heavy chain fusion.

[0015] In some embodiments, the first expression vector is a mammalian expression vector. In some embodiments, the second expression vector is a mammalian expression vector. In some embodiments, the first and second expression vectors are mammalian expression vectors.

[0016] In some embodiments, each heavy chain fusion or Fc fusion is independently a fusion of a heavy chain or Fc with a VH, scFv, or cytokine fused to either the N-terminus or the C-terminus of the heavy chain portion of the heavy chain fusion or Fc portion of the Fc fusion, or between the CH1 and CH2 of the heavy chain portion of the heavy chain fusion. The fusion may be direct or through a linker.

[0017] In some embodiments, each heavy chain fusion is a fusion of a heavy chain with a VH. In some embodiments, each heavy chain fusion is a fusion of a heavy chain with a scFv. In some embodiments, each heavy chain fusion is a fusion of a heavy chain with a cytokine.

[0018] In some embodiments, each heavy chain fusion is a direct fusion of a heavy chain with a VH. In some embodiments, each heavy chain fusion is a direct fusion of a heavy chain with a scFv. In some embodiments, each heavy chain fusion is a direct fusion of a heavy chain with a cytokine.

[0019] In some embodiments, each heavy chain fusion is a fusion of a heavy chain with a VH, wherein the fusion comprises a linker between the heavy chain and the VH. In some embodiments, each heavy chain fusion is a fusion of a heavy chain with a scFv, wherein the fusion comprises a linker between the heavy chain and the scFv. In some embodiments, each heavy chain fusion is a fusion of a heavy chain with a cytokine, wherein the fusion comprises a linker between the heavy chain and the cytokine.

[0020] In some embodiments, each Fc fusion is a fusion of a Fc with a VH. In some embodiments, each Fc fusion is a fusion of a Fc with a scFv. In some embodiments, each Fc fusion is a fusion of a Fc with a cytokine.

[0021] In some embodiments, each Fc fusion is a direct fusion of a Fc with a VH. In some embodiments, each Fc fusion is a direct fusion of a Fc with a scFv. In some embodiments, each Fc fusion is a direct fusion of a Fc with a cytokine.

[0022] In some embodiments, each Fc fusion is a fusion of a Fc with a VH, wherein the fusion comprises a linker between the Fc and the VH. In some embodiments, each Fc fusion is a fusion of a Fc with a scFv, wherein the fusion comprises a linker between the Fc and the scFv. In some embodiments, each Fc fusion is a fusion of a Fc with a cytokine, wherein the fusion comprises a linker between the Fc and the cytokine.

[0023] In some embodiments, the selectable marker of the first expression vector and the selectable marker of the second expression vector are the same. In some embodiments, the selectable marker of the first expression vector and the selectable marker of the second expression vector are different.

[0024] In some embodiments, the selectable marker of the first expression vector is glutamine synthetase or dihydrofolate reductase. In some embodiments, the selectable marker of the second expression vector is glutamine synthetase or dihydrofolate reductase. In certain aspects of these embodiments, the selectable markers of the first expression vector and the second expression vector are both glutamine synthetase.

[0025] In some embodiments, the first, second, and third polyA signal sequences on each expression vector are the same.

[0026] In some embodiments, at least one of the first, second, and third polyA signal sequences on each expression vector is different.

[0027] In some embodiments, the first, second, and third polyA signal sequences on each expression vector are independently selected from the group consisting of a bovine growth hormone (BGH) polyA signal sequence, a thymidine kinase polyA (TKpA) signal sequence, a rabbit beta-globin polyA signal sequence, and a simian virus 40 (SV40) early polyA signal sequence.

[0028] In some embodiments, the first expression vector encodes a heavy chain, and the second expression vector encodes a heavy chain fusion having a VH, scFv, or cytokine fused to the C-terminus of the heavy chain portion of the heavy chain fusion. In some embodiments, the first expression vector encodes a heavy chain, and the second expression vector encodes a heavy chain fusion having a VH fused to the C-terminus of the heavy chain portion of the heavy chain fusion. In some embodiments, the first expression vector encodes a heavy chain, and the second expression vector encodes a heavy chain fusion having a scFv fused to the C-terminus of the heavy chain portion of the heavy chain fusion. In some embodiments, the first expression vector encodes a heavy chain, and the second expression vector encodes a heavy chain fusion having a cytokine fused to the C-terminus of the heavy chain portion of the heavy chain fusion.

[0029] In some embodiments, the first expression vector encodes a heavy chain, and the second expression vector encodes a Fc fusion having a scFv fused to the N-terminus of the Fc portion of the Fc fusion.

[0030] In some embodiments, the first expression vector encodes a heavy chain fusion having a VH or cytokine fused at the C-terminus of the heavy chain portion of the heavy chain fusion, and the second expression vector encodes a heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the heavy chain fusion. In some embodiments, the first expression vector encodes a heavy chain fusion having a VH fused at the C-terminus of the heavy chain portion of the heavy chain fusion, and the second expression vector encodes a heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the heavy chain fusion. In some embodiments, the first expression vector encodes a heavy chain fusion having a cytokine fused at the C-terminus of the heavy chain portion of the heavy chain fusion, and the second expression vector encodes a heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the heavy chain fusion.

[0031] The present disclosure also provides a mammalian host cell comprising any of the expression systems described herein. In certain embodiments, the mammalian host cell is a Chinese hamster ovary (CHO) cell. In certain aspects of this embodiment, the CHO cell is a dihydrofolate reductase deficient (DHFR-) or a glutamine synthetase knockout (GSKO) CHO cell. In some embodiments, the CHO cell is a dihydrofolate reductase deficient (DHFR-) CHO cell. In some embodiments, the CHO cell is a glutamine synthetase knockout (GSKO) CHO cell.

[0032] Additionally, the present disclosure provides a method for producing a multi-specific antibody having only one Fab or having two Fabs that are identical, wherein the method comprises: introducing an expression system described herein into a mammalian host cell; and culturing the mammalian host cell to produce the multi-specific antibody. In some embodiments, the mammalian host cell is a Chinese hamster ovary (CHO) cell. In some embodiments, the CHO cell is a dihydrofolate reductase deficient (DHFR-) CHO cell or a glutamine synthetase knockout (GSKO) CHO cell. In some embodiments, the CHO cell is a dihydrofolate reductase deficient (DHFR-) CHO cell. In some embodiments, the CHO cell is a glutamine synthetase knockout (GSKO) CHO cell.

[0033] The present disclosure further provides a method for preparing a multi-specific antibody having only one Fab or having two Fabs that are identical, wherein the method comprises the steps of: a) introducing two different expression vectors into a host cell, wherein the first expression vector encodes for 1) a light chain and 2) a heavy chain, a heavy chain fusion, or a Fc-fusion; and the second expression vector encodes for 1) the identical light chain, and 2) a heavy chain, a heavy chain fusion, or a Fc-fusion; and b) culturing the host cell in a mammalian cell culture under conditions where the expression vectors express the multi-specific antibody, wherein said multi-specific antibody has only one Fab or has two Fabs that are identical.

[0034] In certain embodiments, each heavy chain fusion or Fc fusion is independently a fusion to a scFv, a VH, or a cytokine. In some embodiments, each heavy chain fusion or Fc fusion is independently a fusion to a scFv. In some embodiments, each heavy chain fusion or Fc fusion is independently a fusion to a VH. In some embodiments, each heavy chain fusion or Fc fusion is independently a fusion to a cytokine.

[0035] In some embodiments, each heavy chain fusion is a fusion of a heavy chain with a VH. In some embodiments, each heavy chain fusion is a fusion of a heavy chain with a scFv. In some embodiments, each heavy chain fusion is a fusion of a heavy chain with a cytokine.

[0036] In some embodiments, each heavy chain fusion is a direct fusion of a heavy chain with a VH. In some embodiments, each heavy chain fusion is a direct fusion of a heavy chain with a scFv. In some embodiments, each heavy chain fusion is a direct fusion of a heavy chain with a cytokine.

[0037] In some embodiments, each heavy chain fusion is a fusion of a heavy chain with a VH, wherein the fusion comprises a linker between the heavy chain and the VH. In some embodiments, each heavy chain fusion is a fusion of a heavy chain with a scFv, wherein the fusion comprises a linker between the heavy chain and the scFv. In some embodiments, each heavy chain fusion is a fusion of a heavy chain with a cytokine, wherein the fusion comprises a linker between the heavy chain and the cytokine.

[0038] In some embodiments, each Fc fusion is a fusion of a Fc with a VH. In some embodiments, each Fc fusion is a fusion of a Fc with a scFv. In some embodiments, each Fc fusion is a fusion of a Fc with a cytokine.

[0039] In some embodiments, each Fc fusion is a direct fusion of a Fc with a VH. In some embodiments, each Fc fusion is a direct fusion of a Fc with a scFv. In some embodiments, each Fc fusion is a direct fusion of a Fc with a cytokine.

[0040] In some embodiments, each Fc fusion is a fusion of a Fc with a VH, wherein the fusion comprises a linker between the Fc and the VH. In some embodiments, each Fc fusion is a fusion of a Fc with a scFv, wherein the fusion comprises a linker between the Fc and the scFv. In some embodiments, each Fc fusion is a fusion of a Fc with a cytokine, wherein the fusion comprises a linker between the Fc and the cytokine.

[0041] In the case where there are two Fabs, although the Fabs are identical, there are one or two heavy chain fusions which can independently have a scFv, VH, or cytokine fused at the N-terminus, C-terminus, between the CH1 and CH2 domains, or any combination of the foregoing. Where more than one heavy chain fusion is present, they can be the same or different.

[0042] In certain embodiments, the multi-specific antibody is a bispecific antibody. In one aspect, the bispecific antibody has 1) a heavy chain; and 2) a heavy chain fusion or Fc fusion with a scFv, a VH, or a cytokine fused to either the N-terminus or the C-terminus of the heavy chain portion of the heavy

chain fusion or the Fc portion of the Fc fusion, or between the CH1 and CH2 of the heavy chain portion of the heavy chain fusion.

[0043] In some embodiments, the multi-specific antibody has only one Fab.

[0044] In some embodiments, the multi-specific antibody has two Fabs that are identical.

[0045] In certain embodiments, the multi-specific antibody is a trispecific antibody. In one aspect, the trispecific antibody has 1) a first heavy chain fusion having a VH or a cytokine fused to either the N-terminus or the C-terminus of the heavy chain portion of the first heavy chain fusion; and 2) a second heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the second heavy chain fusion.

[0046] In some embodiments, the trispecific antibody has 1) a first heavy chain fusion having a VH fused to either the N-terminus or the C-terminus of the heavy chain portion of the first heavy chain fusion; and 2) a second heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the second heavy chain fusion. In some embodiments, the trispecific antibody has 1) a first heavy chain fusion having a VH fused to the N-terminus of the heavy chain portion of the first heavy chain fusion; and 2) a second heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the second heavy chain fusion. In some embodiments, the trispecific antibody has 1) a first heavy chain fusion having a VH fused to the C-terminus of the heavy chain portion of the first heavy chain fusion; and 2) a second heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the second heavy chain fusion.

[0047] In some embodiments, the trispecific antibody has 1) a first heavy chain fusion having a cytokine fused to either the N-terminus or the C-terminus of the heavy chain portion of the first heavy chain fusion; and 2) a second heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the second heavy chain fusion. In some embodiments, the trispecific antibody has 1) a first heavy chain fusion having a cytokine fused to the N-terminus of the heavy chain portion of the first heavy chain fusion; and 2) a second heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the second heavy chain fusion. In some embodiments, the trispecific antibody has 1) a first heavy chain fusion having a cytokine fused to the C-terminus of the heavy chain portion of the first heavy chain fusion; and 2) a second heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the second heavy chain fusion.

[0048] In certain embodiments, each of the expression vectors comprises a selectable marker which can be the same or different. The selectable marker can be selected from the group consisting of glutamine synthetase and dihydrofolate reductase. A promoter operably linked to the nucleotide sequence encoding the selectable marker can be selected from the group consisting of mPGK, SR α , and SV40 promoters.

[0049] In some embodiments, the expression vectors each comprise the same selectable marker. In some embodiments, the expression vectors each comprise a glutamine synthetase selectable marker.

In some embodiments, the expression vectors each comprise a dihydrofolate reductase selectable marker.

[0050] In some embodiments, the expression vectors each comprise a different selectable marker. In some embodiments, one expression vector comprises a glutamine synthetase selectable marker and the other expression vector comprises a dihydrofolate reductase selectable marker.

[0051] In certain embodiments, the host cell is a mammalian host cell. The mammalian host cell can be a Chinese Hamster Ovary (CHO) cell. For example, the CHO cell can be a dihydrofolate reductase deficient (dhfr-) or a glutamine synthetase knockout (GSKO) CHO cell. In some embodiments, the CHO cell is a dihydrofolate reductase deficient (dhfr-) CHO cell. In some embodiments, the CHO cell is a glutamine synthetase knockout (GSKO) CHO cell.

[0052] In certain embodiments, the method further comprises recovering the antibody from the culture. In certain aspects, the recovered antibody modality is purified and formulated in a pharmaceutically acceptable formulation. In some embodiments, a “pharmaceutically acceptable formulation” refers to a formulation that is generally safe, non-toxic, and neither biologically nor otherwise undesirable for use in a subject.

[0053] Further aspects and advantages will be apparent to those of ordinary skill in the art from a review of the following detailed description. The description hereafter includes specific cases, embodiments, and examples with the understanding that the disclosure is illustrative and is not intended to limit the embodiments of the present disclosure to the specific cases, embodiments, and examples described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0054] FIGs. 1A-1B show (A) a schematic of various antibody configurations where the Fab regions are identical or only one Fab is present (left side) and where the Fab regions are different (right side) and (B) a schematic of vector configurations for expression of a single light chain on one vector (top) and for expression of the same light chain on two different vectors (bottom). In the schematic on the top left-hand side of FIG. 1A, the recombinant protein comprises two different heavy chains having the exact same Fab, targeting the same antigen and, on both sides, the Fd and the light chain (LC) are perfect cognate pairs. In the schematic on the top right-hand side of FIG. 1A, the recombinant protein comprises two different heavy chains having different Fabs, targeting two different antigens. In this schematic, the light chain is the same in each Fab, but the Fd is different, meaning that at least one Fd:LC pair is non-cognate (i.e., not a perfect pair).

[0055] FIGs. 2A-2B show (A) a schematic of a C1mAb, and (B) schematics of the vector configurations used in Example 1.

[0056] FIGs. 3A-3B show pool titer in g/L (A) and cell specific productivity (qp) in pg/cell/day (B) from 1xLC and 2xLC vector combinations for a C1mAb-A. Titer and qp were normalized to the 1xLC

0 μ M MSX condition. Titer was measured on day 10 of a fed-batch production. Data is presented as mean values for two independent transfections. Error bars represent the standard deviation (SD) of three technical replicates for fed-batch production. Data is shaded based on vector configurations: one light chain (1x LC) or two light chains (2x LC). MSX concentrations used are indicated.

[0057] FIGs. 4A-4B show normalized effective titer in g/L (A) and nrCE-SDS main peak-% (B) from 1x LC and 2x LC vector combinations for a C1mAb-A. Effective titer was normalized to the 1x LC 0 μ M MSX condition. Normalized effective titer was calculated on day 10 of a fed-batch production. Data is presented as mean values for two independent transfections. Error bars represent the standard deviation (SD) of three technical replicates for fed-batch production. Data is shaded based on vector configurations: one light chain (1x LC) or two light chains (2x LC). MSX concentrations used are indicated.

[0058] FIG. 5 shows a schematic of the vector configurations used in Example 2.

[0059] FIGs. 6A-6B show normalized yield in g/L (A) and nrCE-SDS main peak-% (B) from 1x LC1, 1x LC2, and 2x LC vector combinations for an AmAb-A. Yield was normalized to the 1x LC1 MSX condition. Normalized yield was calculated on day 10 of a fed-batch production.

[0060] FIG. 7 shows a schematic of the vector configurations used in Example 3.

[0061] FIGs. 8A-8B show normalized yield in g/L (A) and nrCE-SDS main peak-% (B) from 1x LC and 2x LC vector combinations for a trispecific antibody. Yield was normalized to the 1x LC1 MSX condition. Normalized yield was calculated on day 10 of a fed-batch production.

[0062] FIG. 9 shows a representative scheme for a C1mAb, asymmetric fusion.

DETAILED DESCRIPTION

[0063] The present disclosure is based, in part, on the discovery that utilizing nucleotide sequences encoding a common or identical light chain on two expression vectors, i.e., having two copies of a light chain coding sequence, with nucleotide sequences encoding two different heavy chains, heavy chain fusions, or Fc-fusions, each on a separate vector, can be used to drive expression of the different antibody chains, thereby increasing the titer of a multi-specific antibody having only one Fab or two identical Fab regions. In contrast to prior methods which produce multi-specific antibodies where the Fabs are different (i.e., have different heavy chain variable regions), the present disclosure provides methods for producing multi-specific antibodies where there is only one Fab or the Fabs are identical. For example, the multi-specific antibody may have only one light chain and an Fc fusion (and therefore only one Fab) or the heavy chains are identical (thereby having two identical Fabs) but at least one heavy chain is modified as a fusion to, for example, a scFv, a cytokine, a VH, etc. By employing bicistronic (not counting the selectable marker) vectors to produce three chains in multi-specific antibodies for expression in host cells to produce antibodies, higher titers can be achieved

compared to expression vectors only having one copy of the light chain (the other expression vector not having nucleotide sequences encoding a light chain).

[0064] Standard antibody production techniques often employ different promoters for the expression of the heavy chain and the light chain to optimize expression of the antibody. This is often necessary because the heavy and light chain are expressed at different levels. The situation becomes even more complicated when the antibody structures contain three chains. Typically, for a three-chain molecule, a common light chain is used along with two unique heavy chains. The light chain is expressed on a bicistronic expression vector along with one of the heavy chains. The other heavy chain is expressed on a monocistronic vector. The inventors have surprisingly found that by including one copy of a common or identical light chain on each expression vector, the titers of the resulting antibody structures can be increased with improved product quality, such as, for example, reduced aggregation, clipping, or presence of non-desired isoforms.

[0065] By employing the methods, expression vectors, and host cells described herein, production of recombinant proteins (e.g., multi-specific antibodies, e.g., three-chain molecules) can be increased while retaining or improving product quality, for example, as assessed by aggregation, clipping, or presence of isoforms. Additionally, biopharmaceuticals may be produced in a less expensive and more consistent manner by employing such methods, expression vectors, and host cells. These innovations find particular utility in the commercial production of antibody modalities having three unique chains.

[0066] The methods described herein employ cell lines (also referred to as “host cells”), preferably mammalian (“mammalian host cells”), grown in cell culture media to produce a recombinant protein of commercial or scientific interest. Cell lines are typically derived from a lineage arising from a primary culture that can be maintained in culture for an unlimited time. Genetically engineering the cell line involves transfecting, transforming, or transducing the cells with two expression vectors where each vector contains nucleotide sequences encoding two antibody chains, having a common light chain, so as to cause the host cell to express an antibody modality having the desired number of chains. Methods and vectors for genetically engineering cells and/or cell lines to express, for example, a protein of interest, are well known to those of skill in the art; for example, various techniques are illustrated in Current Protocols in Molecular Biology, Ausubel *et al.*, eds. (Wiley & Sons, New York, 1988, and quarterly updates); Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Laboratory Press, 1989); Kaufman, R.J., *Large Scale Mammalian Cell Culture*, 1990, pp. 15-69; and Harlow and Lane *Antibodies: A Laboratory Manual* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1990).

DEFINITIONS

[0067] While the terminology used in this application is standard within the art, definitions of certain terms are provided herein to assure clarity and definiteness in the meaning of the claims. Units, prefixes, and symbols may be denoted in their SI (International System of Units) accepted form.

Numeric ranges recited herein are inclusive of the numbers defining the range and include and are supportive of each integer within the defined range. The methods and techniques described herein are generally performed according to conventional methods well-known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated.

[0068] As used herein, the terms “a” and “an” mean one or more unless specifically indicated otherwise. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. Generally, nomenclatures used in connection with, and techniques of, cell and tissue culture, molecular biology, immunology, microbiology, genetics, and protein and nucleic acid chemistry and hybridization described herein are those well-known and commonly used in the art.

[0069] All documents, or portions of documents, cited in this application, including but not limited to patents, patent applications, articles, books, and treatises, are hereby expressly incorporated by reference. What is described in an embodiment of the invention can be combined with other embodiments of the invention.

[0070] The present disclosure provides tools for expressing a “protein of interest,” such as, for example, a multi-specific antibody. A “protein of interest” includes recombinant proteins and engineered proteins (e.g., proteins that do not occur in nature and which have been designed and/or created by humans). A protein of interest can, but need not be, a protein that is known or suspected to be therapeutically relevant.

[0071] As used herein, an “antibody chain” or “chain” refers to antibody light chains, antibody heavy chains, antibody heavy chain fusion proteins, scFv-Fc fusion, VH fusion, cytokine fusions, and the like. The terms “antibody heavy chain” and “antibody light chain” have the standard meaning in the art and include, for example, the various antibody heavy and light chains described elsewhere herein (e.g., heavy and light chains of IgG1, IgG2, IgG3, and IgG4 mAbs). The terms “antibody heavy chain” and “antibody light chain” include standard full-length antibody heavy chains and light chains. The terms “antibody heavy chain fusion” and “antibody heavy chain fusion protein” refer to a polypeptide that contains an antibody heavy chain covalently linked to one or more additional proteins or peptides. For example, an “antibody heavy chain fusion protein” can be an antibody heavy chain covalently linked to a cytokine. By “fusion” or “fused,” it is meant that the linkage may be direct, or via a peptide linker (e.g., a glycine-serine linker). In an antibody heavy chain fusion protein, the antibody heavy chain may be linked to additional protein(s) at the N-terminus or the C-terminus of the heavy chain (or both locations). The antibody heavy chain may also be linked to additional protein sequences, such as an scFv, at an internal amino acid residue or be between the Fab and the Fc. As used herein, an “antibody fusion protein” refers to an antibody as provided herein which is covalently linked to one or more additional proteins or polypeptides (e.g., via a heavy chain or light

chain of the antibody). Thus, an antibody fusion protein contains at least an antibody heavy chain fusion protein or an antibody light chain fusion protein as one of the polypeptides of the antibody fusion protein. Most commonly, an antibody fusion protein is a molecule that contains two antibody light chains, one antibody heavy chain, and one antibody heavy chain fusion protein, such that the additional protein is linked to one of the heavy chains of the antibody.

[0072] For purposes of the expression vector systems, host cells, and methods described herein, a three-chain molecule includes 1) a common or identical light chain (i.e., a single light chain expressed on two bicistronic vectors) and a common heavy chain where one or both of the heavy chains is part of a heavy chain fusion protein, and 2) a light chain bound to only one arm of the antibody, one heavy chain, and a Fc fusion (i.e., heavy chain lacking a Fd region but fused to a protein). An scFv, cytokine, or VH region can be added at either end of a heavy chain or Fc, i.e., at the free end of the variable region or at the free end of the constant region in the case of a heavy chain, or at either end of an Fc. FIG. 1A, on the left side, provides example antibody molecules which can be produced by the methods of the invention. FIG. 1A, on the right side, depicts the common LC approach where the heavy chain variable regions are different.

[0073] As used herein, the terms “polypeptide” and “protein” (e.g., as used in the context of a protein of interest or a polypeptide of interest) are used interchangeably to refer to a polymer of amino acid residues. The terms also apply to amino acid polymers in which one or more amino acid residues is an analog or mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The terms can also encompass amino acid polymers that have been modified, e.g., by the addition of carbohydrate residues to form glycoproteins, or phosphorylated. Polypeptides and proteins can be produced by a naturally-occurring and non-recombinant cell, or polypeptides and proteins can be produced by a genetically-engineered or recombinant cell. Polypeptides and proteins can comprise molecules having the amino acid sequence of a native protein, or molecules having deletions from, additions to, and/or substitutions of one or more amino acids of the native sequence.

[0074] As used herein, the term “heterologous” used in connection with a nucleic acid means having a nucleic acid not naturally occurring within a host cell. This can include mutated sequences, e.g., sequences differing from the naturally occurring sequence. This can include sequences from other species. This can also include having a sequence at a different position in the genome than that naturally occurring in the host cell. This generally does not include natural mutations that may occur in a host cell. A cell already containing a heterologous nucleic acid encoding a protein of interest, for example, by stable integration of an expression cassette, would be considered to contain a heterologous nucleic acid sequence. For clarity, a CHO cell or a derivative thereof (e.g., a DHFR- or GS knockout) having a nucleic acid encoding an antigen-binding protein would be considered to have a heterologous nucleic acid.

[0075] As used herein, the term “operably linked” refers to that the nucleic acid sequences being linked are typically contiguous, or substantially contiguous, and, where necessary to join two protein coding regions, contiguous and in reading frame. However, since enhancers generally function when separated from the promoter by several kilobases and intronic sequences may be of variable lengths, some polynucleotide elements may be operably linked but not contiguous. Two or more nucleic acid sequences may be operably linked in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced.

[0076] As used herein, the term “bioreactor” means any vessel useful for the growth of a cell culture. The cell cultures of the instant disclosure can be grown in a bioreactor, which can be selected based on the application of a protein of interest that is produced by cells growing in the bioreactor. A bioreactor can be of any size so long as it is useful for the culturing of cells; typically, a bioreactor is sized appropriate to the volume of cell culture being grown inside of it. Typically, a bioreactor will be at least 1 liter and may be 2, 5, 10, 50, 100, 200, 250, 500, 1,000, 1500, 2000, 2,500, 5,000, 8,000, 10,000, 12,000 liters or more, or any volume in between. The internal conditions of the bioreactor, including, but not limited to, pH and temperature, can be controlled during the culturing period. Those of ordinary skill in the art will be aware of, and will be able to select, suitable bioreactors for use in practicing the methods disclosed herein based on the relevant considerations.

[0077] As used herein, “cell culture” or “culture” is meant to refer to the growth and propagation of cells outside of a multicellular organism or tissue. Suitable culture conditions for mammalian cells are known in the art. See, e.g., *Animal cell culture: A Practical Approach*, D. Rickwood, ed., Oxford University Press, New York (1992). Mammalian cells may be cultured in suspension or while attached to a solid substrate. Fluidized bed bioreactors, hollow fiber bioreactors, roller bottles, shake flasks, or stirred tank bioreactors, with or without microcarriers, can be used. 500L to 2000L bioreactors can be used as well as 1000L to 2000L bioreactors.

[0078] The term “cell culture medium” (also called “culture medium,” “cell culture media,” or “tissue culture media”) refers to any nutrient solution used for growing cells, e.g., animal or mammalian cells, and which generally provides at least one or more components from the following: an energy source (usually in the form of a carbohydrate such as glucose); one or more of all essential amino acids, and generally the twenty basic amino acids, plus cysteine; vitamins and/or other organic compounds typically required at low concentrations; lipids or free fatty acids; and trace elements, e.g., inorganic compounds or naturally occurring elements that are typically required at very low concentrations, usually in the micromolar range.

[0079] The nutrient solution may optionally be supplemented with additional optional components to optimize growth of cells, such as hormones and other growth factors, e.g., transferrin, epidermal growth factor, insulin, insulin-like growth factor, serum, and the like; salts, e.g., calcium, magnesium and phosphate, and buffers, e.g., HEPES; nucleosides and bases, e.g., adenosine, thymidine,

hypoxanthine; and protein and tissue hydrolysates, e.g., hydrolyzed animal or plant protein (peptone or peptone mixtures, which can be obtained from animal byproducts, purified gelatin or plant material); antibiotics, e.g., gentamycin; anti-clumping agents; cell protectants or surfactants such as Pluronic® F68 (also referred to as Lutrol® F68 and Kolliphor® P188; nonionic triblock composed of a central hydrophobic chain of polyoxypropylene (poly(propylene oxide)) flanked by two hydrophilic chains of polyoxyethylene (poly(ethylene oxide))); polyamines, e.g., putrescine, spermidine and spermine (see, e.g., International Patent Application Publication No. WO 2008/154014) and pyruvate (see, e.g. U.S. Pat. No. 8,053,238) depending on the requirements of the cells to be cultured and/or the desired cell culture parameters.

[0080] Cell culture media include those that are typically employed in and/or are known for use with any cell culture process, such as, but not limited to, batch, extended batch, fed-batch, and/or perfusion or continuous culturing of cells.

[0081] A “base” (or batch) cell culture medium refers to a cell culture medium that is typically used to initiate a cell culture and is sufficiently complete to support the cell culture.

[0082] A “fed-batch culture” refers to a form of suspension culture and means a method of culturing cells in which additional components are provided to the culture at a time or times subsequent to the beginning of the culture process. The provided components typically comprise nutritional supplements for the cells which have been depleted during the culturing process. Additionally, or alternatively, the additional components may include supplementary components (e.g., a cell-cycle inhibitory compound). A fed-batch culture is typically stopped at some point and the cells and/or components in the medium are harvested and optionally purified.

[0083] A “growth” cell culture medium refers to a cell culture medium that is typically used in cell cultures during a period of exponential growth, a “growth phase,” and is sufficiently complete to support the cell culture during this phase. A growth cell culture medium may also contain selection agents that confer resistance or survival to selectable markers incorporated into the host cell line. Such selection agents include, but are not limited to, geneticin (G418), blasticidin, neomycin, hygromycin B, puromycin, zeocin, methionine sulfoximine, methotrexate, glutamine-free cell culture medium, cell culture medium lacking glycine, hypoxanthine and thymidine, or thymidine alone.

[0084] A “perfusion” cell culture medium refers to a cell culture medium that is typically used in cell cultures that are maintained by perfusion or continuous culture methods and is sufficiently complete to support the cell culture during this process. Perfusion cell culture medium formulations may be richer or more concentrated than base cell culture medium formulations to accommodate the method used to remove the spent medium. Perfusion cell culture medium can be used during both the growth and production phases.

[0085] A “production” cell culture medium refers to a cell culture medium that is typically used in cell cultures during the transition when exponential growth is ending and protein production takes

over, “transition” and/or “product” phases, and is sufficiently complete to maintain a desired cell density, viability and/or product titer during this phase.

[0086] Concentrated cell culture medium can contain some or all of the nutrients necessary to maintain the cell culture; in particular, concentrated medium can contain nutrients identified as or known to be consumed during the course of the production phase of the cell culture. Concentrated medium may be based on just about any cell culture media formulation. Such a concentrated feed medium can contain some or all the components of the cell culture medium at, for example, about 2X, 3X, 4X, 5X, 6X, 7X, 8X, 9X, 10X, 12X, 14X, 16X, 20X, 30X, 50X, 100X, 200X, 400X, 600X, 800X, or even about 1000X of their normal amount.

[0087] The components used to prepare cell culture medium may be completely milled into a powder medium formulation; partially milled with liquid supplements added to the cell culture medium as needed; or added in a completely liquid form to the cell culture.

[0088] Cell cultures can also be supplemented with independent concentrated feeds of particular nutrients which may be difficult to formulate or are quickly depleted in cell cultures. Such nutrients may be amino acids such as tyrosine, cysteine and/or cystine (see e.g., International Patent Application Publication No. WO2012/145682). The independent feeds can begin prior to or at the start of the production phase. The independent feeds can be accomplished by fed batch to the cell culture medium on the same or different days as the concentrated feed medium. The independent feeds can also be perfused on the same or different days as the perfused medium.

[0089] “Serum-free” applies to a cell culture medium that does not contain animal sera, such as fetal bovine serum. Various tissue culture media, including defined culture media, are commercially available, for example, any one or a combination of the following cell culture media can be used: RPMI-1640 Medium, RPMI-1641 Medium, Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium Eagle, F-12K Medium, Ham's F12 Medium, Iscove's Modified Dulbecco's Medium, McCoy's 5A Medium, Leibovitz's L-15 Medium, and serum-free media such as EX-CELL™ 300 Series (JRH Biosciences, Lenexa, Kansas), MCDB 302 (Sigma Aldrich Corp., St. Louis, MO), among others. Serum-free versions of such culture media are also available. Cell culture media may be supplemented with additional or increased concentrations of components such as amino acids, salts, sugars, vitamins, hormones, growth factors, buffers, antibiotics, lipids, trace elements and the like, depending on the requirements of the cells to be cultured and/or the desired cell culture parameters. Customized cell culture media can also be used.

[0090] “Titer” means the total amount of a polypeptide or protein of interest (which may be a naturally occurring or recombinant protein of interest) produced by a cell culture in a given amount of medium volume. Titer can be expressed in units of milligrams or micrograms of polypeptide or protein per milliliter (or other measure of volume) of medium. “Cumulative titer” is the titer produced

by the cells during the course of the culture, and can be determined, for example, by measuring daily titers and using those values to calculate the cumulative titer.

[0091] As used herein, the term “host cell” is understood to include a cell that has been genetically engineered to express a polypeptide of interest. Genetically engineering a cell involves transfecting, transforming, or transducing the cell with a nucleic acid encoding a recombinant polynucleotide molecule (a “gene of interest”), and/or otherwise altering (e.g., by homologous recombination and gene activation or fusion of a recombinant cell with a non-recombinant cell) so as to cause the host cell to express a desired recombinant polypeptide. Methods and vectors for genetically engineering cells and/or cell lines to express a polypeptide of interest are well known to those of skill in the art; for example, various techniques are illustrated in *Current Protocols in Molecular Biology*, Ausubel *et al.*, eds. (Wiley & Sons, New York, 1988, and quarterly updates); Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Laboratory Press, 1989); Kaufman, R.J., *Large Scale Mammalian Cell Culture*, 1990, pp. 15-69. The term includes the progeny of the parent cell, whether or not the progeny is identical in morphology or in genetic makeup to the original parent cell, so long as the gene of interest is present. A cell culture can comprise one or more host cells.

[0092] It is understood that wherever embodiments are described herein with the language “comprising,” otherwise analogous embodiments described in terms of “consisting of” and/or “consisting essentially of” are also provided.

EXPRESSION VECTOR CONFIGURATIONS

[0093] A typical antibody is a Y-shaped molecule having four polypeptide chains – two identical heavy chains and two identical light chains. Such an antibody is preferably expressed from a single vector. However, bispecific antibodies having three chains require the use of alternative formats and are typically expressed from two different vectors. See, e.g., Spiess *et al.*, 2015, *Mol. Immunol.* 67:95-106; Brinkmann *et al.*, 2017, *MAbs* 9:192-212; and Ma *et al.*, 2021, *Frontiers in Immunology* 12:626616.

[0094] Expression vectors will typically include one or more promoters that are recognized by the host organism and are operably linked to the nucleotide sequence encoding a protein of interest. Promoters are untranscribed sequences located upstream (i.e., 5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control transcription of the structural gene.

[0095] An expression system for a three-chain antibody has typically comprised 2 expression vectors with 1) a first vector having a first promoter driving expression of a first nucleotide sequence encoding a common antibody light chain and a second promoter driving expression of a second nucleotide sequence encoding a first antibody heavy chain (and a promoter driving expression of a coding sequence encoding a selectable marker) and 2) a second vector having a third promoter driving expression of a second antibody heavy chain (and a promoter driving expression of a coding sequence encoding a selectable marker). This vector configuration is depicted in FIG. 1B as a 1x LC vector

configuration. In some embodiments of the present disclosure, the term “expression system” may be replaced by “expression vector system.”

[0096] The present disclosure provides an expression system for a three-chain antibody which comprises 2 expression vectors with 1) a first vector having a first promoter driving expression of a first nucleotide sequence encoding a common or identical antibody light chain and a second promoter driving expression of a heavy chain, heavy chain fusion, or Fc fusion (and a third promoter driving expression of a coding sequence encoding a selectable marker) and 2) a second vector having a first promoter driving expression of a first nucleotide sequence encoding the common or identical antibody light chain and a second promoter driving expression of a heavy chain, heavy chain fusion, or Fc fusion (and a third promoter driving expression of a coding sequence encoding a selectable marker). This vector configuration is depicted in FIG. 1B as an exemplary 2x LC vector configuration. Such three-chain antibodies/molecules may include 1) a common or identical light chain (i.e., a single light chain expressed on two bicistronic vectors) and a common heavy chain where one or both of the heavy chains is a heavy chain fusion protein, and 2) a light chain bound to only one arm of the antibody, one heavy chain, and one heavy chain lacking a Fd region. An scFv, cytokine, or VH/VHH region can be added at either end of heavy chain, i.e., at the free end of the variable region or at the free end of the constant region.

[0097] The exemplary 2x LC vector configuration described above can be modified with different promoter choices. For example, a single promoter can be used for the common light chain and both heavy chains, i.e., the first promoter and the second promoter on each expression vector are the same. In some embodiments, the first promoter on each vector is the same, and the second promoter on each vector is the same, but the first promoter and second promoters are different.

[0098] Promoters of particular interest for nucleotide sequences encoding antibody chains include the human cytomegalovirus IE1 gene promoter enhancer (CMV) (Boshart et al., 1985, Cell 41:521-30; GenBank Accession No. X03922) and hamster glyceraldehyde-3-phosphate dehydrogenase promoter and intron (GAPDH) (U.S. Patent No. 10,202,261). Additional sequences can also be combined with promoters to improve expression. An example of such a sequence is the adenovirus tripartite leader (ADL). (See Gingeras et al., 1982, J. Biol. Chem. 257:13475-91; GenBank Accession No. J01917).

[0099] In some embodiments, the first promoter on the first expression vector is a GAPDH promoter. In some embodiments, the second promoter on the first expression vector is a GAPDH promoter. In some embodiments, the first promoter on the first expression vector is a GAPDH promoter, and the second promoter on the first expression vector is a GAPDH promoter.

[0100] In some embodiments, the first promoter on the second expression vector is a GAPDH promoter. In some embodiments, the second promoter on the second expression vector is a GAPDH promoter. In some embodiments, the first promoter on the second expression vector is a GAPDH promoter, and the second promoter on the second expression vector is a GAPDH promoter.

[0101] In some embodiments, the first promoter on the first expression vector is a GAPDH promoter, the second promoter on the first expression vector is a GAPDH promoter, the first promoter on the second expression vector is a GAPDH promoter, and the second promoter on the second expression vector is a GAPDH promoter.

[0102] In some embodiments, the first promoter and/or the second promoter of each expression vector is a combination of the CMV promoter enhancer and GAPDH (CMV/GAPDH). In this combination, both the CMV promoter enhancer and GAPDH promoter are operably linked to a nucleotide sequence, and the combination may function as a better promoter than the GAPDH promoter alone. In certain embodiments, the CMV promoter is 5' of the GAPDH. Collectively, CMV/GAPDH is a promoter.

[0103] In some embodiments, the CMV/GAPDH promoter comprises a polynucleotide sequence that is 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the polynucleotide sequence of SEQ ID NO: 1. In some embodiments, the CMV/GAPDH promoter comprises the polynucleotide sequence of SEQ ID NO: 1.

[0104] In some embodiments, the first promoter on the first expression vector is a CMV/GAPDH promoter. In some embodiments, the second promoter on the first expression vector is a CMV/GAPDH promoter. In some embodiments, the first promoter on the first expression vector is a CMV/GAPDH promoter, and the second promoter on the first expression vector is a CMV/GAPDH promoter.

[0105] In some embodiments, the first promoter on the second expression vector is a CMV/GAPDH promoter. In some embodiments, the second promoter on the second expression vector is a CMV/GAPDH promoter. In some embodiments, the first promoter on the second expression vector is a CMV/GAPDH promoter, and the second promoter on the second expression vector is a CMV/GAPDH promoter.

[0106] In some embodiments, the first promoter on the first expression vector is a CMV/GAPDH promoter, the second promoter on the first expression vector is a CMV/GAPDH promoter, the first promoter on the second expression vector is a CMV/GAPDH promoter, and the second promoter on the second expression vector is a CMV/GAPDH promoter.

[0107] In some embodiments, the first promoter on the second expression vector is a CMV promoter. In some embodiments, the second promoter on the second expression vector is a CMV promoter. In some embodiments, the first promoter on the second expression vector is a CMV promoter, and the second promoter on the second expression vector is a CMV promoter.

[0108] In some embodiments, the first promoter on the first expression vector is a CMV promoter, the second promoter on the first expression vector is a CMV promoter, the first promoter on the second expression vector is a CMV promoter, and the second promoter on the second expression vector is a CMV promoter.

[0109] In embodiments described herein, the heavy chains (HC1 and HC2) are different but either 1) have identical heavy chain Fd regions or 2) one heavy chain lacks the Fd region (i.e., is a Fc). Thus, in situation 1), when paired with the common light chain, the different heavy chains have the same Fab targeting the same antigen. While the heavy chains have identical heavy chain variable regions, one or both heavy chains are fused to an additional amino acid sequence. Such additional amino acid sequence can be a heavy chain variable region, an scFv, or a cytokine. In situation 2), one heavy chain does not form a Fab as one of the chains lacks the Fd region. In both situations, there is only one Fab type in the molecule.

[0110] In the expression vectors described above, a polyA signal sequence can follow each gene (i.e., the coding sequences encoding the first and second antibody chains and the selectable marker). PolyA signal sequences are known in the art and include a bovine growth hormone (BGH) polyA signal sequence (e.g., Pfarr et al., 1986, DNA, 5(2):115-22; Goodwin and Rottman, 1992, J. Biol. Chem., 267(23):16330-16334), a thymidine kinase polyA (TKpA) signal sequence (Cole and Stacy, 1985, Mol Cell Biol., 5(8):2104-13), a rabbit beta-globin polyA signal sequence (Lanoix et al., 1988; EMBO J. 7(8):2515-22; GenBank Accession No. MG356850.1), and a simian virus 40 (SV40) early polyA signal sequence (Connelly and Manley, 1988, Genes Dev., 2(4):440-52; GenBank Accession No. J02400). In some embodiments, the first, second, and third polyA signal sequences on each expression vector are independently selected from the group consisting of a bovine growth hormone (BGH) polyA signal sequence, a thymidine kinase polyA (TKpA) signal sequence, a rabbit beta-globin polyA signal sequence, and a simian virus 40 (SV40) early polyA signal sequence.

[0111] In some embodiments, the first, second, and third polyA signal sequences on each expression vector are a bovine growth hormone (BGH) polyA signal sequence.

[0112] In some embodiments, the first, second, and third polyA signal sequences on each expression vector are a thymidine kinase polyA (TKpA) signal sequence.

[0113] In some embodiments, the first, second, and third polyA signal sequences on each expression vector are a rabbit beta-globin polyA signal sequence.

[0114] In some embodiments, the first, second, and third polyA signal sequences on each expression vector are a simian virus 40 (SV40) early polyA signal sequence.

[0115] In some embodiments, the BGH polyA signal sequence comprises a polynucleotide sequence that is 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the polynucleotide sequence of SEQ ID NO: 5. In some embodiments, the BGH polyA signal sequence comprises the polynucleotide sequence of SEQ ID NO: 5.

[0116] In some embodiments, the rabbit beta-globin polyA signal sequence comprises a polynucleotide sequence that is 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the polynucleotide sequence of SEQ ID NO: 6. In some embodiments, the rabbit beta-globin polyA signal sequence comprises the polynucleotide sequence of SEQ ID NO: 6.

[0117] In some embodiments, the SV40 early polyA signal sequence comprises a polynucleotide sequence that is 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the polynucleotide sequence of SEQ ID NO: 7. In some embodiments, the SV40 early polyA signal sequence comprises the polynucleotide sequence of SEQ ID NO: 7.

[0118] In some embodiments, the thymidine kinase polyA (TKpA) signal sequence comprises a polynucleotide sequence that is 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the polynucleotide sequence of SEQ ID NO: 8. In some embodiments, the thymidine kinase polyA (TKpA) signal sequence comprises the polynucleotide sequence of SEQ ID NO: 8.

[0119] In one embodiment, the antibody modality produced from the methods described herein is a C1mAb (Fab-heteroFc-[scFv*], asymmetric fusion or a {scFv-Fab*Fab]-heteroFc-[protein]). For example, for a three-chain modality, one expression cassette contains coding sequences for one heavy chain and one light chain and the other expression cassette contains coding sequences for a heavy chain-scFv fusion and the common light chain. The first antibody chain in each expression cassette can be either the light chain or the heavy chain/heavy chain-scFv fusion. In one aspect, the first antibody chain is the light chain and the second antibody chain is either a heavy chain or a heavy chain-scFv fusion.

[0120] A representative scheme for a C1mAb, asymmetric fusion, is shown in FIG. 9.

[0121] The expression vectors provided herein provide optimal expression of three-chain antibodies, possibly due to optimal chain ratios of the expressed polypeptides. Chain ratios can be measured using techniques well-known in the art.

[0122] It has been found that expressing a common or identical light chain on both expression vectors for a multi-specific antibody, e.g., a three-chain antibody, having identical heavy chain variable regions surprisingly results in higher production of the antibody.

SELECTABLE MARKERS

[0123] For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker is generally introduced into the host cells in the same expression vector as the gene(s) of interest.

[0124] A selectable marker gene encodes a protein necessary for the survival and growth of a host cell grown in a selective culture medium. Typical selection marker genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, tetracycline, or kanamycin for prokaryotic host cells; (b) complement auxotrophic deficiencies of the cell; or (c) supply critical nutrients not available from complex or defined media through metabolism. Specific antibiotic-resistance selectable markers are the kanamycin resistance gene, the ampicillin resistance gene, the tetracycline resistance gene, and the neomycin resistance gene.

[0125] Other selectable genes may be used to amplify the gene that will be expressed. Amplification is the process wherein genes that are required for production of a protein critical for growth or cell survival are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Examples of suitable selectable markers for mammalian cells include, but are not limited to, glutamine synthetase (GS), dihydrofolate reductase (DHFR), asparaginase (Aspg; see Ha et al., *Biotechnol Bioeng.* 2023 120:1159-1166), and promoterless thymidine kinase genes.

[0126] Mammalian cell transformants are placed under selection pressure wherein only the transformants are uniquely adapted to survive by virtue of the selectable gene present in the vector. Selection pressure is imposed by culturing the transformed cells under conditions in which the concentration of selection agent in the medium is successively increased, thereby leading to additional stringency and/or amplification of both the selectable gene and the DNA that encodes a protein of interest. As a result, increased quantities of a polypeptide of interest are synthesized from the amplified DNA. The selection agent for GS is methionine sulfoximine (MSX). The selection agent for DHFR is methotrexate (MTX).

[0127] Relative to DHFR-based systems, GS knockout cell lines (GSKO) provide sufficient selection stringency without MSX or with low MSX concentrations, while 25 μ M MSX coupled with the GS-knockout cell line led to higher selection efficiency compared with CHOK1SV cell lines at higher MSX concentrations (Fan et al., 2012, *Biotechnol Bioeng.*, 109(4):1007-1015). A previous report showed that increasing the MSX concentration in the seed train stage after clone selection increased productivity without significant impacts on cell growth, GS and target gene copy numbers and expression, and maintained product quality attributes in multiple GS knockout cell lines (Tian et al., 2020, *Engineering in Life Sciences* 20(3-4):112-125). Chain/vector expression can be influenced by increasing stringency during pool recovery/selection by adding MSX.

[0128] In certain embodiments, the MSX concentration can be optimized for one of the promoters driving GS expression. In a specific embodiment, the MSX concentration is optimized for the GS linked to the more difficult to express chain.

[0129] In certain embodiments, the selectable marker in an expression vector (e.g., the first expression vector and/or the second expression vector) is glutamine synthetase. Glutamine synthetase (GS) catalyzes glutamine biosynthesis by the condensation of ammonia with glutamate.

[0130] In certain embodiments, the promoter SR α is operably linked to the selectable marker. In other embodiments, the promoter mPGK is operably linked to the selectable marker. Other suitable promoters can be selected from those well-known in the art.

[0131] As described above, the present disclosure provides an expression system comprising: 1) a first expression vector which comprises a polynucleotide sequence which comprises the following elements in 5' to 3' order: a) a first promoter operably linked to a nucleotide sequence encoding a light chain followed by a polyadenylation (polyA) signal sequence; b) a second promoter operably

linked to a nucleotide sequence encoding a heavy chain, a heavy chain fusion, or a Fc fusion followed by a second polyA signal sequence; and c) a third promoter operably linked to a nucleotide sequence encoding a selectable marker followed by a third polyA signal sequence; and 2) a second expression vector which comprises a polynucleotide sequence which comprises the following elements in 5' to 3' order: a) a first promoter operably linked to a nucleotide sequence encoding an identical copy of the light chain followed by a polyA signal sequence; b) a second promoter operably linked to a nucleotide sequence encoding a heavy chain, a heavy chain fusion, or a Fc fusion followed by a second polyA signal sequence; and c) a third promoter operably linked to a nucleotide sequence encoding a selectable marker followed by a third polyA signal sequence, wherein, when expressed in a host cell, the expression system produces a multi-specific antibody that has only one Fab or has two Fabs that are identical.

[0132] In some embodiments, the selectable marker of the first expression vector is glutamine synthetase. In some embodiments, the selectable marker of the second expression vector is glutamine synthetase. In some embodiments, the selectable marker of the first expression vector is glutamine synthetase, and the selectable marker of the second expression vector is glutamine synthetase.

[0133] In some embodiments, the third promoter on the first expression vector is a SR α promoter. In some embodiments, the third promoter on the second expression vector is a SR α promoter. In some embodiments, the third promoter on the first expression vector is a SR α promoter, and the third promoter on the second expression vector is a SR α promoter.

[0134] In some embodiments, the third promoter on the first expression vector is a mPGK promoter. In some embodiments, the third promoter on the second expression vector is a mPGK promoter. In some embodiments, the third promoter on the first expression vector is a mPGK promoter, and the third promoter on the second expression vector is a mPGK promoter.

[0135] In some embodiments, the third promoter on the first expression vector is a SV40 promoter. In some embodiments, the third promoter on the second expression vector is a SV40 promoter. In some embodiments, the third promoter on the first expression vector is a SV40 promoter, and the third promoter on the second expression vector is a SV40 promoter.

[0136] In some embodiments, the third promoter on the first expression vector is a SR α promoter, and the third promoter on the second expression vector is a mPGK promoter. In some embodiments, the third promoter on the first expression vector is a mPGK promoter, and the third promoter on the second expression vector is a SR α promoter.

[0137] In some embodiments, the third promoter on the first expression vector is a SR α promoter, and the selectable marker of the first expression vector is glutamine synthetase. In some embodiments, the third promoter on the second expression vector is a SR α promoter, and the selectable marker of the second expression vector is glutamine synthetase. In some embodiments, the third promoter on the first expression vector is a SR α promoter, the third promoter on the second

expression vector is a SR α promoter, the selectable marker of the first expression vector is glutamine synthetase, and the selectable marker of the second expression vector is glutamine synthetase.

[0138] In some embodiments, the SR α promoter comprises a polynucleotide sequence that is 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the polynucleotide sequence of SEQ ID NO: 2. In some embodiments, the SR α promoter comprises the polynucleotide sequence of SEQ ID NO: 2.

[0139] In some embodiments, the mPGK promoter comprises a polynucleotide sequence that is 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the polynucleotide sequence of SEQ ID NO: 3. In some embodiments, the mPGK promoter comprises the polynucleotide sequence of SEQ ID NO: 3.

[0140] In some embodiments, the SV40 promoter comprises a polynucleotide sequence that is 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the polynucleotide sequence of SEQ ID NO: 4. In some embodiments, the SV40 promoter comprises the polynucleotide sequence of SEQ ID NO: 4.

ADDITIONAL EXPRESSION VECTOR COMPONENTS

[0141] The expression vectors described herein are useful for transformation of a host cell and can contain additional nucleic acid sequences for plasmid maintenance and for cloning and expression of exogenous nucleotide sequences. Such sequences will typically include one or more of the following nucleotide sequences (in addition to the promoter(s)/enhancer fragment(s), antibody chains, selectable marker(s), and other sequences (e.g., polyadenylation sequences) specifically described above): one or more enhancer sequences, an origin of replication, transcriptional and translational control sequences, a transcriptional termination sequence, a complete intron sequence containing a donor and acceptor splice site, various pre- or pro-sequences to improve glycosylation or yield, a native or heterologous signal sequence (leader sequence or signal peptide) for polypeptide secretion, a ribosome binding site, a polyadenylation sequence, internal ribosome entry site (IRES) sequences, an expression augmenting sequence element (EASE), tripartite leader (TPL) and VA gene RNAs from Adenovirus 2, and a polylinker region for inserting the polynucleotide encoding the polypeptide to be expressed. Vectors may be constructed from a starting vector such as a commercially available vector, additional elements may be individually obtained and ligated into the vector. Methods used for obtaining each of the components are well known to one skilled in the art.

[0142] Vector components may be homologous (i.e., from the same species and/or strain as the host cell), heterologous (e.g., from a species other than the host cell species or strain), hybrid (i.e., a combination of flanking sequences from more than one source), synthetic or native. The sequences of components useful in the vectors may be obtained by methods well-known in the art, such as those previously identified by mapping and/or by restriction endonuclease. In addition, they can be obtained by polymerase chain reaction (PCR) and/or by screening a genomic library with suitable probes.

[0143] A ribosome-binding site is usually necessary for translation initiation of mRNA and is characterized by a Shine-Dalgarno sequence (prokaryotes) or a Kozak sequence (eukaryotes). The

element is typically located 3' to the promoter and 5' to the coding sequence of the polypeptide to be expressed.

[0144] An origin of replication aids in the amplification of the vector in a host cell. They may be included as part of commercially available prokaryotic vectors and may also be chemically synthesized based on a known sequence and ligated into the vector. Various viral origins (e.g., SV40, polyoma, adenovirus, vesicular stomatitis virus (VSV), or papillomaviruses such as HPV or BPV) are useful for cloning vectors in mammalian cells.

[0145] Transcriptional and translational control sequences for mammalian host cell expression vectors can be excised from viral genomes. Commonly used enhancer sequences are derived from polyoma virus, adenovirus 2, simian virus 40 (SV40), and human cytomegalovirus (CMV). For example, the human CMV promoter/enhancer of immediate early gene 1 may be used. (See, e.g., Patterson *et al.*, 1994, Applied Microbiol. Biotechnol. 40:691-98.) DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites can be used to provide other genetic elements for expression of a structural gene sequence in a mammalian host cell. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment, which can also contain a viral origin of replication (Fiers *et al.*, 1978, Nature 273:113; Kaufman, 1990, Meth. in Enzymol. 185:487-511). Smaller or larger SV40 fragments can also be used, provided the approximately 250 bp sequence extending from the Hind III site toward the BglII site located in the SV40 viral origin of replication site is included. For examples, an enhancer sequence may be inserted into the vector to increase transcription by higher eukaryotes. Enhancers are cis-acting elements of DNA, usually about 10-300 bp in length, that act on the promoter to increase transcription. Enhancers are relatively orientation and position independent, having been found at positions both 5' and 3' to the transcription unit. Several enhancer sequences available from mammalian genes are known (e.g., globin, elastase, albumin, alpha-feto-protein and insulin). Typically, however, an enhancer from a virus is used. The SV40 enhancer, the cytomegalovirus early promoter enhancer, the polyoma enhancer, and adenovirus enhancers known in the art are exemplary enhancing elements for the activation of eukaryotic promoters. While an enhancer may be positioned in the vector either 5' or 3' to a coding sequence, it is typically located at a site 5' from the promoter.

[0146] In some cases, such as where glycosylation is desired in a eukaryotic host cell expression system, one may manipulate the various pre- or pro-sequences to improve glycosylation or yield. For example, one may alter the peptidase cleavage site of a particular signal peptide, or add prosequences, which also may affect glycosylation. The final protein product may have, in the -1 position (relative to the first amino acid of the mature protein), one or more additional amino acids incident to expression, which may not have been totally removed. For example, the final protein product may have one or two amino acid residues found in the peptidase cleavage site, attached to the amino-

terminus. Alternatively, use of some enzyme cleavage sites may result in a slightly truncated form of the desired polypeptide if the enzyme cuts at such area within the mature polypeptide.

[0147] A sequence encoding an appropriate native or heterologous signal sequence (leader sequence or signal peptide) can be incorporated into an expression vector, to promote extracellular secretion of the protein of interest. The choice of signal peptide or leader depends on the type of host cells in which the protein of interest to be produced, and a heterologous signal sequence can replace the native signal sequence. Non-limiting examples of signal peptides that are functional in mammalian host cells include the following: the signal sequence for interleukin-7 described in U.S. Patent No. 4,965,195; the signal sequence for interleukin-2 receptor described in Cosman et al., 1984, Nature 312:768; the interleukin-4 receptor signal peptide described in EP Patent No. 0367 566; the type I interleukin-1 receptor signal peptide described in U.S. Pat. No. 4,968,607; the type II interleukin-1 receptor signal peptide described in EP Patent No. 0 460 846.

[0148] Additional control sequences shown to improve expression of heterologous genes from mammalian expression vectors include such elements as the expression augmenting sequence element (EASE) derived from CHO cells (Morris et al., in Animal Cell Technology, pp. 529-534 (1997); U.S. Patent Nos. 6,312,951 B1, 6,027,915, and 6,309,841 B1) and the tripartite leader (TPL) and VA gene RNAs from Adenovirus 2 (Gingeras *et al.*, 1982, J. Biol. Chem. 257:13475-13491). The internal ribosome entry site (IRES) sequences of viral origin allows bicistronic mRNAs to be translated efficiently (Oh and Samow, 1993, Current Opinion in Genetics and Development 3:295-300; Ramesh *et al.*, 1996, Nucleic Acids Research 24:2697-2700).

[0149] Vectors may be selected to be functional in the particular host cell employed (i.e., the vector is compatible with the host cell machinery, permitting amplification and/or expression of the gene can occur). In some embodiments, vectors are used that employ protein-fragment complementation assays using protein reporters, such as dihydrofolate reductase (see, for example, U.S. Patent No. 6,270,964). Suitable expression vectors are known in the art and are also commercially available.

[0150] TABLE 1 provides non-limiting example synthetic nucleotide (DNA) sequences for certain expression vector components that may be used in expression vectors of the present disclosure.

TABLE 1. Non-Limiting Example Expression Vector Component Sequences

Description	SEQ ID NO:	Nucleotide Sequence
CMV/GADPH Promoter	1	GTTGACATTGATTATTGACTAGTTATTAATAGTAATCAAT TACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCG CGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACC GCCCCAACGACCCCCGCCCATTGACGTCAATAATGACGT ATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGAC GTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTG GCAGTACATCAAGTGTATCATATGCCAAGTCCGCCCCCT ATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTAT GCCCAGTACATGACCTTACGGGACTTTCCTACTTGGCA

Description	SEQ ID NO:	Nucleotide Sequence
		GTACATCTACGTATTAGTCATCGCTATTACCATGGCCTCC AGACCACGAGAAGATCCTCAACTTTTCCACAGCCTTTG CATAAAGGGGAGAGGGTCGGCGGTGCAGCTGTGGCAC ACACGCACTTCTGCTCAACCCGCCCCCCCCCGCCCCG TTCTGTTCCTTCCCAGGTTCTCCCCATTTTATCGGGGC GGCAACTTTTAGGTCCCTGGGTCTGGAAGTCCTTAGT ACACACTCTTCGTCTTAAGTCCATAGTCTGTATTCCCT CGGTCTATCCTGTCCCCATCACCGGGTCACCTCCCCA GCGAAGCAATCTCAGTTCCCCTCCCCCTCTCAGCCCCG AGCCACACGTTTGGTGCCTGCACATTTCAAAAACGAG GCGGGTCCAAAGAGAGGGGGTGGGGAGGTGCCGAGTG GCCCAGCTACTCGCGGCTTTACGGGTGCACGTAGTCA GGCCTCAGCGCCCTTGAGCTGTGACTGGATGGATGAGC GGGGCGGGAGGCGGGGCGAGCGTCTCGGCGCTCCCC ACCACCCAGTTCCTATATAAGCAGAGCTCGTTTAGTGA ACCGTCAGATCCTCACTCTCTTCCGCATCGCTGTCTGCG AGGGCCAGCTGTTGGGCTCGCGGTTGAGGACAACTC TTCGCGGTCTTTCCAGTACTCTTGATCGGAAACCCGTC GGCCTCCGAACGGTACTCCGCCACCGAGGGACCTGAG CGAGTCCGCATCGACCGGATCGGAAAACCTCTCGAGAA AGGCGTCTAACCAGTCACAGTCGCAAGGTAGGCTGAG CACCGTGGCGGGCGGCAGCGGGTGGCGGTGGGGTTG TTTCTGGCGGAGGTGCTGCTGATGATGTAATTAAAGTAG GCGCGGATGGTCGAGGTGAGGTGTGGCAGGCTTGAGA TCCAGCTGTTGGGGTGAGTACTCCCTCTCAAAAGCGGG CATTACTTCTGCGCTAAGATTGTCAGTTTCCAAAACGA GGAGGATTTGATATTCACCTGGCCCGATCTGGCCATACA CTTGAGTGACAATGACATCCACTTTGCCTTTCTCTCCAC AGGTGTCCACTCCAG
SR α Promoter	2	CTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCC AGGCTCCCCAGCAGGCAGAAAGTATGCAAAGCATGCATC TCAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGC TCCCCAGCAGGCAGAAAGTATGCAAAGCATGCATCTCAA TTAGTCAGCAACCATAGTCCCGCCCCCTAACTCCGCCCAT CCGCCCCCTAACTCCGCCAGTTCCGCCCATTTCTCCGCC CCATGGCTGACTAATTTTTTTTATTTATGCAGAGGCCGA GGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAG GAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAAGCTG GTCGAGGCTCGCATCTCTCCTTCACGCGCCCGCCGCC TACCTGAGGCCGCCATCCACGCCGGTTGAGTCGCGTTC TGCCGCCTCCCGCCTGTGGTGCCTCCTGAACTGCGTCC GCCGTCTAGGTAAGTTTAAAGCTCAGGTCGAGACCGGG CCTTTGTCCGGCGCTCCCTTGGAGCCTACCTAGACTCA GCCGGCTCTCCACGCTTTGCCTGACCCTGCTTGCTCAA CTCTACGTCTTTGTTTCGTTTTCTGTTCTGCGCCGTTACA GATCCGTCGAGGAAGTGA AAAAACCAGAAAGTTAACTG GTAAGTTTAGTCTTTTTGTCTTTTATTTACAGTCCCGGAT CCGGTGGTGGTGCAAATCAAAGAACTGCTCCTCAGTGG ATGTTGCCTTTACTTCTAGGCCTGTACGGAAGTGTTACT TCTGCTCTAAAAGCTGCTGCAACAAGCTTCTA
mPGK Promoter	3	TTCTACCGGGTAGGGGAGGCGCTTTTCCCAAGGCAGTC TGGAGCATGCGCTTAGCAGCCCCGCTGGGCACTTGGC GCTACACAAGTGGCCTCTGGCCTCGCACACATTCCACA

Description	SEQ ID NO:	Nucleotide Sequence
		TCCACCGGTAGGCGCCAACCGGCTCCGTTCTTTGGTGG CCCCCTCGCGCCACCTTCTACTCCTCCCCTAGTCAGGAA GTTCCCCCCCCGCCCCGAGCTCGCGTCGTGCAGGACGT GACAAATGGAAGTAGCACCTCTCACTAGTCTCGTGCAG ATGGACAGCACCGCTGAGCAATGGAAGCGGGTAGGCC TTTGGGGCAGCGGCCAATAGCAGCTTTGCTCCTTCGCT TTCTGGGCTCAGAGGCTGGGAAGGGGTGGGTCCGGGG GCGGGCTCAGGGGCGGGCTCAGGGGCGGGGCGGGCGC CCGAAGGTCTCCGAGGCCCCGGCATTCTGCACGCTTC AAAAGCGCACGTCTGCCGCGCTGTTCTCTCTTCTCAT CTCCGGGCCTTTTCA
SV40 Promoter	4	GGAATGTGTGTCTAGTTAGGGTGTGGAAAGTCCCCAGGC TCCCCAGCAGGCAGAGAAGTATGCAAAGCATGCATCTCAA TTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCC CAGCAGGCAGAGAAGTATGCAAAGCATGCATCTCAATTAG TCAGCAACCATAGTCCCGCCCCCTAACTCCGCCCCATCCCG CCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCAT GGCTGACTAATTTTTTTTTATTTATGCAGAGGCCGAGGCC GCCTCTGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGG CTTTTTTGAGGCCTAGCTTTTGCAAAAAGCT
Bovine Growth Hormone (BGH) polyA	5	CTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTGCCCT CCCCCGTGCCTTCCTTGACCCCTGGAAGGTGCCACTCCC ACTGTCTCTTTCCTAATAAAATGAGGAAATTGCATCGCAT TGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTG GGGCAGGACAGCAAGGGGGGAGGATTGGGAAGACAATA GCAGGCATGCTGGGGATGCGGTGGGCTCTATGG
Rabbit Beta-Globin polyA	6	TCTGGCTAATAAAGGAAATTTATTTTCATTGCAATAGTGT GTTGGAATTTTTTGTGTCTCTCACTCGGAAGGACATATG GGAGGGCAAATCATT
SV40 early polyA	7	AACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGC AATAGCATCACAAATTTACAAATAAAGCATTTTTTTTCA CTGCATTCTAGTTGTGGTTTGTCCAACTCATCAATGTA TCTTATCATGTCTG
Thymidine Kinase polyA	8	GGGGGAGGCTAACTGAAACACGGAAGGAGACAATACC GGAAGGAACCCGCGCTATGACGGCAATAAAAAGACAG AATAAACGCACGGGTGTTGGGTCGTTTGTTTATAAAC GCGGGGTTTCGGTCCCAGGGCTGGCACTCTGTCTGATACC CCACCGAGACCCCATTTGGGGCCAATACGCCCCGCTTTC TTCTTTTCCCCACCCACCCCCCAAGTTCGGGTGAAG GCCCAGGGCTCGCAGCCAACGTCGGGGCGGCAGGCC TGCCATAGC

PROTEINS OF INTEREST

[0151] The present disclosure provides expression systems comprising: 1) a first expression vector which comprises a polynucleotide sequence which comprises the following elements in 5' to 3' order:

- a) a first promoter operably linked to a nucleotide sequence encoding a light chain followed by a polyadenylation (polyA) signal sequence; b) a second promoter operably linked to a nucleotide sequence encoding a heavy chain, a heavy chain fusion, or a Fc fusion followed by a second polyA

signal sequence; and c) a third promoter operably linked to a nucleotide sequence encoding a selectable marker followed by a third polyA signal sequence; and 2) a second expression vector which comprises a polynucleotide sequence which comprises the following elements in 5' to 3' order: a) a first promoter operably linked to a nucleotide sequence encoding an identical copy of the light chain followed by a polyA signal sequence; b) a second promoter operably linked to a nucleotide sequence encoding a heavy chain, a heavy chain fusion, or a Fc fusion followed by a second polyA signal sequence; and c) a third promoter operably linked to a nucleotide sequence encoding a selectable marker followed by a third polyA signal sequence. When these expression systems are expressed in a host cell (e.g., a CHO cell), they produce specific proteins of interest, i.e., multi-specific antibodies that have only one Fab or have two Fabs that are identical.

[0152] Polypeptides and proteins of interest produced using the described expression systems can be of scientific or commercial interest, including protein-based therapeutics. Proteins of interest may include, among other things, secreted proteins, non-secreted proteins, intracellular proteins, or membrane-bound proteins. Polypeptides and proteins of interest can be produced by recombinant animal cell lines using cell culture methods and may be referred to as "recombinant proteins." The expressed protein(s) may be produced intracellularly or secreted into the culture medium from which it can be recovered and/or collected. The term "isolated protein" or "isolated recombinant protein" refers to a polypeptide or protein of interest, which is purified away from proteins or polypeptides or other contaminants that would interfere with its therapeutic, diagnostic, prophylactic, research or other use. Proteins of interest include proteins that exert a therapeutic effect by binding a target, particularly a target among those listed below, including targets derived therefrom, targets related thereto, and modifications thereof.

[0153] Proteins of interest include "antigen-binding proteins," in particular "antibody modalities." Antigen-binding protein refers to proteins or polypeptides that comprise an antigen-binding region or antigen-binding portion that has affinity for another molecule to which it binds (antigen). Antigen-binding proteins encompass antibodies, peptibodies, antibody fragments, antibody derivatives, antibody analogs, fusion proteins (including single-chain variable fragments (scFvs), double-chain (divalent) scFvs, and IgGscFv (see, e.g., Orcutt *et al.*, 2010, Protein Eng Des2 Sel 23:221-228), hetero-IgG (see, e.g., Liu *et al.*, 2015, J Biol Chem 290:7535-7562), muteins, and XmAb® (Xencor, Inc., Monrovia, CA) molecules. Non-limiting examples of antigen-binding proteins include a human antibody, a humanized antibody, a chimeric antibody, a recombinant antibody, a single chain antibody; a diabody; a triabody; a tetrabody; a Fab fragment; a F(ab')₂ fragment; an IgD antibody; an IgE antibody; an IgM antibody; an IgG1 antibody; an IgG2 antibody; an IgG3 antibody; or an IgG4 antibody, and fragments thereof. Also included are bispecific T cell engagers (BiTE®), bispecific T cell engagers having extensions, such as half-life extensions, for example HLE BiTE molecules,

HeteroIg BITE molecules, and others. Some of these antigen-binding protein formats may be produced using expression vector systems of the present disclosure.

[0154] As used herein, the term “antigen-binding protein” is used in its broadest sense and means a protein comprising a portion that binds to an antigen or target and, optionally, a scaffold or framework portion that allows the antigen binding portion to adopt a conformation that promotes binding of the antigen binding protein to the antigen. The antigen binding protein can comprise, for example, an alternative protein scaffold or artificial scaffold with grafted CDRs or CDR derivatives. Such scaffolds include, but are not limited to, antibody-derived scaffolds comprising mutations introduced to, for example, stabilize the three-dimensional structure of the antigen binding protein as well as wholly synthetic scaffolds comprising, for example, a biocompatible polymer. *See, e.g.*, Korndorfer *et al.*, 2003, *Proteins: Structure, Function, and Bioinformatics*, 53(1):121-129; Roque *et al.*, 2004, *Biotechnol. Prog.* 20:639-654. In addition, peptide antibody mimetics (“PAMs”) can be used, as well as scaffolds based on antibody mimetics utilizing fibronectin components as a scaffold.

[0155] An antigen-binding protein can have, for example, the structure of a naturally occurring immunoglobulin. An “immunoglobulin” is a tetrameric molecule. In a naturally occurring immunoglobulin, each tetramer is composed of two identical pairs of polypeptide chains, each pair having one “light” (about 25 kDa) and one “heavy” chain (about 50-70 kDa). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function. Human light chains are classified as kappa and lambda light chains. Heavy chains are classified as mu, delta, gamma, alpha, or epsilon, and define the antibody’s isotype as IgM, IgD, IgG, IgA, and IgE, respectively.

[0156] Naturally occurring immunoglobulin chains exhibit the same general structure of relatively conserved framework regions (FR) joined by three hypervariable regions, also called complementarity determining regions or CDRs. From N-terminus to C-terminus, both light and heavy chains comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. The assignment of amino acids to each domain can be done in accordance with the definitions of Kabat *et al.* in *Sequences of Proteins of Immunological Interest*, 5th Ed., US Dept. of Health and Human Services, PHS, NIH, NIH Publication no. 91-3242, (1991). As desired, the CDRs can also be redefined according to an alternative nomenclature scheme, such as that of Chothia (see Chothia and Lesk, 1987, *J. Mol. Biol.* 196:901-917; Chothia *et al.*, 1989, *Nature* 342:878-883 or Honegger and Pluckthun, 2001, *J. Mol. Biol.* 309:657-670).

[0157] In the context of the instant disclosure, an antigen-binding protein is said to “specifically bind” or “selectively bind” its target antigen when the dissociation constant (K_D) is $\leq 10^{-8}$ M. The antibody specifically binds antigen with “high affinity” when the K_D is $\leq 5 \times 10^{-9}$ M, and with “very high affinity” when the K_D is $\leq 5 \times 10^{-10}$ M.

[0158] The term “antibody” includes reference to both glycosylated and non-glycosylated immunoglobulins of any isotype or subclass or to an antigen-binding region thereof that competes with the intact antibody for specific binding, unless otherwise specified. Additionally, the term “antibody” refers to an intact immunoglobulin that competes with the intact antibody for specific binding, unless otherwise specified. Unless otherwise specified, antibodies include human, humanized, chimeric, multi-specific, monoclonal, polyclonal, heteroIgG, bispecific, and oligomers. Antibodies include the IgG1-, IgG2- IgG3- or IgG4-type.

[0159] An antigen-binding fragment includes Fab, Fab', F(ab')₂, Fv, diabodies, Fd, dAb, maxibodies, single chain antibody molecules, single domain V_HH, complementarity determining region (CDR) fragments, scFv, diabodies, triabodies, tetrabodies, and polypeptides that contain at least a portion of an immunoglobulin that is sufficient to confer specific antigen binding to a target polypeptide.

[0160] An antigen-binding protein can have one or more binding sites. If there is more than one binding site, the binding sites can be identical to one another or can be different. For example, a naturally occurring human immunoglobulin typically has two identical binding sites, while a “bispecific” or “bifunctional” antibody has two different binding sites. One standard nomenclature for multi-specific antibody modalities is VERITAS. See Biswas et al., 2023, mAbs 15:1-9.

[0161] A Fab fragment is a monovalent fragment having the V_L, V_H, C_L and C_H1 domains; a F(ab')₂ fragment is a bivalent fragment having two Fab fragments linked by a disulfide bridge at the hinge region; a Fd fragment has the V_H and C_H1 domains; an Fv fragment has the V_L and V_H domains of a single arm of an antibody; and a dAb fragment has a V_H domain, a V_L domain, or an antigen-binding fragment of a V_H or V_L domain (U.S. Pat. Nos. 6,846,634, 6,696,245, U.S. Patent Application Publication Nos. 2005/0202512, 2004/0202995, 2004/0038291, 2004/0009507, 2003/0039958, Ward et al., 1989, *Nature* 341:544-546).

[0162] A single-chain antibody (scFv) is an antibody in which a V_L and a V_H region are joined via a linker (e.g., a synthetic sequence of amino acid residues) to form a continuous protein chain wherein the linker is long enough to allow the protein chain to fold back on itself and form a monovalent antigen binding site (*see, e.g.*, Bird et al., 1988, *Science* 242:423-26 and Huston et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:5879-83, U.S. Patent Nos. 7,741,465, and 6,319,494 as well as Eshhar et al., 1997, *Cancer Immunol Immunotherapy* 45:131-136). An scFv retains the parent antibody's ability to specifically interact with target antigen.

[0163] A protein of interest can comprise a human antibody. The term “human antibody” includes all antibodies that have one or more variable and constant regions derived from human immunoglobulin sequences. In one embodiment, all of the variable and constant domains are derived from human immunoglobulin sequences (a fully human antibody). Such antibodies can be prepared in a variety of ways, including through the immunization with an antigen of interest of a mouse that is genetically modified to express antibodies derived from human heavy and/or light chain-encoding genes, such as

a mouse derived from a Xenomouse[®], UltiMab[™], or Velocimmune[®] system, or a rat derived from UniRat[®]. Phage-based approaches can also be employed.

[0164] Alternatively, a protein of interest can comprise a humanized antibody. A “humanized antibody” has a sequence that differs from the sequence of an antibody derived from a non-human species by one or more amino acid substitutions, deletions, and/or additions, such that the humanized antibody is less likely to induce an immune response, and/or induces a less severe immune response, as compared to the non-human species antibody, when it is administered to a human subject. In one embodiment, certain amino acids in the framework and constant domains of the heavy and/or light chains of the non-human species antibody are mutated to produce the humanized antibody. In another embodiment, the constant domain(s) from a human antibody are fused to the variable domain(s) of a non-human species. Examples of how to make humanized antibodies can be found in U.S. Pat. Nos. 6,054,297, 5,886,152 and 5,877,293.

[0165] Also included are modified proteins, such as are proteins modified chemically by a non-covalent bond, covalent bond, or both a covalent and non-covalent bond. Also included are proteins further comprising one or more post-translational modifications, which may be made by cellular modification systems or modifications introduced *ex vivo* by enzymatic and/or chemical methods or introduced in other ways.

[0166] Proteins of interest may also include recombinant fusion proteins comprising, for example, a multimerization domain, such as a leucine zipper, a coiled coil, an Fc portion of an immunoglobulin, and the like. Also included are proteins comprising all or part of the amino acid sequences of differentiation antigens (referred to as CD proteins) or their ligands or proteins substantially similar to either of these.

[0167] In some embodiments, proteins of interest may include proteins that bind specifically to one or more CD proteins, HER receptor family proteins, cell adhesion molecules, growth factors, nerve growth factors, fibroblast growth factors, transforming growth factors (TGF), insulin-like growth factors, osteoinductive factors, insulin and insulin-related proteins, coagulation and coagulation-related proteins, colony stimulating factors (CSFs), other blood and serum proteins blood group antigens; receptors, receptor-associated proteins, growth hormones, growth hormone receptors, T-cell receptors; neurotrophic factors, neurotrophins, relaxins, interferons, interleukins, viral antigens, lipoproteins, integrins, rheumatoid factors, immunotoxins, surface membrane proteins, transport proteins, homing receptors, addressins, regulatory proteins, and immunoadhesins.

[0168] In some embodiments, proteins of interest bind to one of more of the following, alone or in any combination: CD proteins including but not limited to CD3, CD4, CD5, CD7, CD8, CD19, CD20, CD22, CD25, CD30, CD33, CD34, CD38, CD40, CD70, CD123, CD133, CD138, CD171, and CD174, HER receptor family proteins, including, for instance, HER2, HER3, HER4, and the EGF receptor, EGFRvIII, cell adhesion molecules, for example, LFA-1, Mol, p150,95, VLA-4, ICAM-1,

VCAM, and alpha v/beta 3 integrin, growth factors, including but not limited to, for example, vascular endothelial growth factor ("VEGF"); VEGFR2, growth hormone, thyroid stimulating hormone, follicle stimulating hormone, luteinizing hormone, growth hormone releasing factor, parathyroid hormone, mullerian-inhibiting substance, human macrophage inflammatory protein (MIP-1-alpha), erythropoietin (EPO), nerve growth factor, such as NGF-beta, platelet-derived growth factor (PDGF), fibroblast growth factors, including, for instance, aFGF and bFGF, epidermal growth factor (EGF), Cripto, transforming growth factors (TGF), including, among others, TGF- α and TGF- β , including TGF- β 1, TGF- β 2, TGF- β 3, TGF- β 4, or TGF- β 5, insulin-like growth factors-I and -II (IGF-I and IGF-II), des(1-3)-IGF-I (brain IGF-I), and osteoinductive factors, insulins and insulin-related proteins, including but not limited to insulin, insulin A-chain, insulin B-chain, proinsulin, and insulin-like growth factor binding proteins; (coagulation and coagulation-related proteins, such as, among others, factor VIII, tissue factor, von Willebrand factor, protein C, alpha-1-antitrypsin, plasminogen activators, such as urokinase and tissue plasminogen activator ("t-PA"), bombazine, thrombin, thrombopoietin, and thrombopoietin receptor, colony stimulating factors (CSFs), including the following, among others, M-CSF, GM-CSF, and G-CSF, other blood and serum proteins, including but not limited to albumin, IgE, and blood group antigens, receptors and receptor-associated proteins, including, for example, flk2/flt3 receptor, obesity (OB) receptor, growth hormone receptors, and T-cell receptors; neurotrophic factors, including but not limited to, bone-derived neurotrophic factor (BDNF) and neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6); relaxin A-chain, relaxin B-chain, and prorelaxin, interferons, including for example, interferon-alpha, -beta, and -gamma, interleukins (ILs), e.g., IL-1 to IL-10, IL-12, IL-15, IL-17, IL-23, IL-12/IL-23, IL-2Ra, IL-1-R1, IL-6 receptor, IL-4 receptor and/or IL-13 to the receptor, IL-13RA2, or IL-17 receptor, IL-1RAP; viral antigens, including but not limited to, an AIDS envelope viral antigen, lipoproteins, calcitonin, glucagon, atrial natriuretic factor, lung surfactant, tumor necrosis factor-alpha and -beta, enkephalinase, BCMA, IgKappa, ROR-1, ERBB2, mesothelin, RANTES (regulated on activation normally T-cell expressed and secreted), mouse gonadotropin-associated peptide, DNase, FR-alpha, inhibin, and activin, integrin, protein A or D, rheumatoid factors, immunotoxins, bone morphogenetic protein (BMP), superoxide dismutase, surface membrane proteins, decay accelerating factor (DAF), AIDS envelope, transport proteins, homing receptors, MIC (MIC-a, MIC-B), ULBP 1-6, EPCAM, addressins, regulatory proteins, immunoadhesins, antigen-binding proteins, somatropin, CTGF, CTLA4, eotaxin-1, MUC1, CEA, c-MET, Claudin-18, GPC-3, EPHA2, FPA, LMP1, MG7, NY-ESO-1, PSCA, ganglioside GD2, ganglioside GM2, BAFF, OPGL (RANKL), myostatin, Dickkopf-1 (DKK-1), Ang2, NGF, IGF-1 receptor, hepatocyte growth factor (HGF), TRAIL-R2, c-Kit, B7RP-1, PSMA, NKG2D-1, programmed cell death protein 1 and ligand, PD1 and PDL1, mannose receptor/hCG β , hepatitis-C virus, mesothelin dsFv[PE38] conjugate, Legionella pneumophila (lly), IFN gamma, interferon gamma induced protein 10 (IP10), IFNAR, TALL-1, thymic stromal

lymphopoietin (TSLP), proprotein convertase subtilisin/Kexin Type 9 (PCSK9), stem cell factors, Flt-3, calcitonin gene-related peptide (CGRP), OX40L, $\alpha 4\beta 7$, platelet specific (platelet glycoprotein IIb/IIIb (PAC-1), transforming growth factor beta (TGF β), Zona pellucida sperm-binding protein 3 (ZP-3), TWEAK, platelet derived growth factor receptor alpha (PDGFR α), sclerostin, and biologically active fragments or variants of any of the foregoing.

[0169] A “Fc region,” as the term is used herein, comprises two heavy chain fragments comprising the C_H2 and C_H3 domains of an antibody. The two heavy chain fragments are held together by two or more disulfide bonds and by hydrophobic interactions of the C_H3 domains. A “Fc” is one heavy chain fragment of a Fc region.

GENERATION OF MAMMALIAN HOST CELLS EXPRESSING A PROTEIN OF INTEREST

[0170] Expression of a protein of interest in a cell can be achieved by well-known methods, either transiently or by stable expression (Davis et al., Basic Methods in Molecular Biology, 2nd ed., Appleton & Lange, Norwalk, Conn., 1994; Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001).

[0171] Methods for stable integration are well-known in the art. Briefly, stable integration is commonly achieved by transiently introducing a heterologous polynucleotide or a vector containing the heterologous polynucleotide into the host cell, which facilitates the stable integration of said heterologous polynucleotide into the cell genome. Typically, the heterologous polynucleotide is flanked by homology arms, i.e., sequences homologous to the region upstream and downstream to the integration site. Before their introduction into the mammalian host cell, circular vectors may be linearized to facilitate integration into the cell genome. Methods for the introduction of vectors into cells are well known in the art and include transfection with biological methods, such as viral delivery, with chemical methods, such as using cationic polymers, calcium phosphate, cationic lipids or cationic amino acids; with physical methods, such as electroporation or microinjection; or with mixed approaches, such as protoplast fusion.

[0172] A specific method of stable integration uses recombinase mediated cassette exchange (RMCE; Bode and Baer, 2001, Curr Opin Biotechnol. 12:473-80, and Bode et al., 2000, Biol. Chem. 381:801- 813) for site-specific integration in the genome (also termed “targeted integration”). Site-specific recombinases such as Flp and Cre mediate recombination between two copies of their target sequence termed FRT and loxP, respectively. The use of two incompatible target sequences, for example FRT in combination with F3 (Schlake and Bode, 1994, Biochemistry, 33:12746-51), as well as inverted recognition target sites (Feng et al., 1999, J. Mol. Biol. 292:779-85), allows the insertion of DNA segments into a predefined chromosomal locus carrying target sequences in a similar configuration. See also EP Patent No. EP1781796B1 and EP Patent Application Publication No. EP2789691A1.

[0173] Insertion of RMCE into a specific site in the genome can be mediated by nucleases (e.g., zinc finger protein (ZFP), transcription activator-like effector nuclease (TALEN), clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)) that can be engineered to create single- and double-stranded breaks (SSBs/DSBs) in the genome. There are two major and distinct pathways to repair DSBs -- homologous recombination and non-homologous end-joining (NHEJ). Homologous recombination requires the presence of a homologous sequence as a template (e.g., "donor" containing RMCE) to guide the cellular repair process and the results of the repair are error-free and predictable. In the absence of a template (or "donor") sequence for homologous recombination, the cell typically attempts to repair the DSB via the unpredictable and error-prone process of non-homologous end-joining (NHEJ).

[0174] A vector may be any molecule or entity (e.g., nucleic acid, plasmid, bacteriophage, transposon, cosmid, chromosome, virus, virus capsid, virion, naked DNA, complexed DNA and the like) suitable for use to transfer and/or transport protein encoding information into a host cell and/or to a specific location and/or compartment within a host cell. Vectors can include viral and non-viral vectors, non-episomal mammalian vectors. Vectors are often referred to as expression vectors, for example, recombinant expression vectors and cloning vectors. This disclosure provides specific examples of expression vector systems useful for expressing multi-specific molecules, e.g., three-chain molecules, such as molecules having identical heavy chain variable regions. The expression vector systems of the present disclosure may be introduced into a host cell to allow replication of the vector(s) and thereby amplify the copies of the polynucleotide(s) contained therein. As described above, cloning vectors may contain sequence components generally include, without limitation, an origin of replication, promoter sequences, transcription initiation sequences, enhancer sequences, and selectable markers. These elements may be selected as appropriate by a person of ordinary skill in the art.

[0175] Following construction, one or more vectors may be inserted into a suitable cell for amplification and/or polypeptide expression. The transformation of an expression vector into a selected cell may be accomplished by well-known methods including transfection, infection, calcium phosphate co-precipitation, electroporation, nucleofection, microinjection, DEAE-dextran mediated transfection, cationic lipids mediated delivery, liposome mediated transfection, microprojectile bombardment, receptor-mediated gene delivery, delivery mediated by polylysine, histone, chitosan, and peptides. The method selected will in part be a function of the type of host cell to be used. These methods and other suitable methods are well known to the skilled artisan and are set forth in manuals and other technical publications, for example, in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2001).

[0176] The term "transformation" refers to a change in a cell's genetic characteristics, and a cell has been transformed when it has been modified to contain new DNA or RNA. For example, a cell is

transformed where it is genetically modified from its native state by introducing new genetic material via transfection, transduction, or other techniques. Following transfection or transduction, the transforming DNA can recombine with that of the cell by physically integrating into a chromosome of the cell or can be maintained transiently as an episomal element without being replicated, or can replicate independently as a plasmid. A cell is considered to have been “stably transformed” when the transforming DNA is replicated with the division of the cell.

[0177] The term “transfection” refers to the uptake of foreign or exogenous DNA by a cell. A number of transfection techniques are well known in the art and are disclosed herein. See, *e.g.*, Graham *et al.*, 1973, *Virology* 52:456; Sambrook *et al.*, 2001, *Molecular Cloning: A Laboratory Manual*, supra; Davis *et al.*, 1986, *Basic Methods in Molecular Biology*, Elsevier; Chu *et al.*, 1981, *Gene* 13:197.

[0178] The term “transduction” refers to the process whereby foreign DNA is introduced into a cell via viral vector. See Jones *et al.*, (1998). *Genetics: principles and analysis*. Boston: Jones & Bartlett Publ.

CELL LINES

[0179] In the methods disclosed herein, any mammalian cell line can be used, with CHO cell lines being one preferred example of a cell line that may be used in combination with the expression vector systems of the present disclosure. A wide variety of mammalian cell lines suitable for growth in culture are available from the American Type Culture Collection (Manassas, Va.) and commercial vendors. Non-limiting examples of cell lines commonly used in the industry include monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, (Graham *et al.*, 1977, *J. Gen Virol.* 36:59); baby hamster kidney cells (BHK, ATCC CCL 10); mouse Sertoli cells (TM4, Mather, 1980, *Biol. Reprod.* 23:243-251); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HeLa, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human hepatoma cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, 1982, *Annals N.Y Acad. Sci.* 383:44-68); MRC 5 cells or FS4 cells; mammalian myeloma cells, and a number of other cell lines and Chinese hamster ovary (CHO) cells.

[0180] Large-scale production of proteins for commercial applications is typically carried out in suspension culture. Therefore, mammalian host cells used to generate the recombinant mammalian cells described herein can, but need not be, adapted to growth in suspension culture. A variety of host cells adapted to growth in suspension culture are known, including mouse myeloma NS0 cells and CHO cells from CHO-S, DG44, and DXB11 cell lines. Other suitable cell lines include, but are not limited to, mouse myeloma SP2/0 cells, baby hamster kidney BHK-21 cells, human PER.C6® cells,

human embryonic kidney HEK-293 cells, and cell lines derived or engineered from any of the cell lines disclosed herein.

[0181] CHO cells are widely used to produce complex recombinant proteins, including CHOK1 cells (ATCC CCL61). The dihydrofolate reductase (DHFR)-deficient mutant cell lines (Urlaub *et al.*, 1980, *Proc Natl Acad Sci USA* 77: 4216-4220), DXB11 and DG-44, are desirable CHO host cell lines because the efficient DHFR selectable and amplifiable gene expression system allows high level recombinant protein expression in these cells (Kaufman R. J., 1990, *Meth Enzymol* 185:537-566). Also included are the glutamine synthase (GS)-knockout CHOK1SV cell lines, making use of glutamine synthetase (GS)-based methionine sulfoximine (MSX) selection. Other suitable CHO host cells could include, but are not limited to, the following (ECACC accession numbers in brackets): CHO (85050302), CHO (PROTEIN FREE) (00102307), CHO-K1 (85051005), CHO-K1/SF (93061607), CHO/DHFR-(94060607), CHO/DHFR-AC-free (05011002), RR-CHOK1 (92052129).

CELL CULTURE PROCESSES

[0182] Host cells transfected with the vector systems described herein may be cultured in adherent culture or suspension cultures grown in stirred tank reactors (including traditional batch and fed-batch cell cultures, which may but need not comprise a spin filter), perfusion systems (including alternating tangential flow (“ATF”) cultures, acoustic perfusion systems, depth filter perfusion systems, and other systems), hollow fiber bioreactors (HFB, which in some cases may be employed in perfusion processes) as well as various other cell culture methods (see, e.g., Tao *et al.*, 2003, *Biotechnol. Bioeng.* 82:751-65; Kuystermans & Al-Rubeai, (2011) “Bioreactor Systems for Producing Antibody from Mammalian Cells” in Antibody Expression and Production, Cell Engineering 7:25-52, Al-Rubeai (ed) Springer; Catapano *et al.*, (2009) “Bioreactor Design and Scale-Up” in Cell and Tissue Reaction Engineering: Principles and Practice, Eibl *et al.* (eds) Springer-Verlag, incorporated herein by reference in their entirety).

[0183] During recombinant protein production, it is desirable to have a controlled system where cells are grown to a desired density and then the physiological state of the cells is switched to a growth-arrested, high productivity state where the cells use energy and substrates to produce the recombinant protein of interest instead of making more cells. Various methods for accomplishing this goal exist, and include temperature shifts and amino acid starvation, as well as use of a cell-cycle inhibitor or other molecule that can arrest cell growth without causing cell death.

[0184] The production of a recombinant protein begins with establishing a mammalian cell production culture of cells that express the protein, in a culture plate, flask, tube, bioreactor or other suitable vessel. Suitable bioreactors volumes include, but are not limited to, 500L, 1000L, 2000L, 5000L, 10000L, up to 20000L. The seed cell density used to inoculate the bioreactor can have a positive impact on the level of recombinant protein produced. In one embodiment, the bioreactor is

inoculated with at least 0.5×10^6 , 1.0×10^6 , 2.0×10^6 , 3.0×10^6 , 5.0×10^6 or 10×10^6 viable cells/mL in a serum-free culture medium.

[0185] The mammalian cells then undergo an exponential growth phase. The cell culture can be maintained without supplemental feeding until a desired cell density is achieved. In one embodiment, the cell culture is maintained for up to three days with or without supplemental feeding. In another embodiment, the culture can be inoculated at a desired cell density to begin the production phase without a brief growth phase. In any of the embodiments herein, the switch from the growth phase to production phase can also be initiated by any of the aforementioned methods.

[0186] Three methods are typically used in commercial processes for the production of recombinant proteins by mammalian cell culture: batch culture, fed-batch culture, and perfusion culture. Batch culture is a discontinuous method where cells are grown in a fixed volume of culture media for a short period of time followed by a full harvest. Cultures grown using the batch method experience an increase in cell density until a maximum cell density is reached, followed by a decline in viable cell density as the media components are consumed and levels of metabolic by-products (such as lactate and ammonia) accumulate. Harvest typically occurs at the point when the maximum cell density is achieved (e.g., 5×10^6 cells/mL or greater, depending on media formulation, cell line, etc.). The batch process is the simplest culture method; however, viable cell density is limited by the nutrient availability and once the cells are at maximum density, the culture declines and production decreases. There is no ability to extend a production phase because the accumulation of waste products and nutrient depletion rapidly lead to culture decline (typically around 3 to 7 days).

[0187] Fed-batch culture improves on the batch process by providing bolus or continuous media feeds to replenish those media components that have been consumed. Since fed-batch cultures receive additional nutrients throughout the run, they have the potential to achieve higher cell densities (>10 to 30×10^6 cells/mL, depending on media formulation, cell line, etc.) and increased product titers, when compared to the batch method. Unlike the batch process, a biphasic culture can be created and sustained by manipulating feeding strategies and media formulations to distinguish the period of cell proliferation to achieve a desired cell density (the growth phase) from the period of suspended or slow cell growth (the production phase). As such, fed batch cultures have the potential to achieve higher product titers compared to batch cultures. Typically, a batch method is used during the growth phase and a fed-batch method used during the production phase, but a fed-batch feeding strategy can be used throughout the entire process. However, unlike the batch process, bioreactor volume is a limiting factor which limits the amount of feed. Also, as with the batch method, metabolic by-product accumulation will lead to culture decline, which limits the duration of the production phase, about 10 to 21 days. Fed-batch cultures are discontinuous, and harvest typically occurs when metabolic by-product levels or culture viability reach predetermined levels. When compared to a batch culture, in

which no feeding occurs, a fed batch culture can produce greater amounts of recombinant protein. See e.g., U.S. Patent No. 5,672,502.

[0188] Perfusion culture is one in which the cell culture receives fresh perfusion feed medium while simultaneously removing spent medium. Perfusion can be continuous, stepwise, intermittent, or a combination of any or all of any of these. Perfusion rates can be less than a working volume to many working volumes per day. The cells are retained in the culture and the spent medium that is removed is substantially free of cells or has significantly fewer cells than the culture. Recombinant proteins expressed by the cell culture can also be retained in the culture. Perfusion can be accomplished by a number of means including centrifugation, sedimentation, or filtration, *See e.g.* Voisard *et al.*, 2003, *Biotechnology and Bioengineering* 82:751-65. An example of a filtration method is alternating tangential flow filtration. Alternating tangential flow is maintained by pumping medium through hollow-fiber filter modules. *See e.g.* US Patent No. 6,544,424; Furey, 2002, *Gen. Eng. News*. 22 (7):62-63.

[0189] “Perfusion flow rate” is the amount of media that is passed through (added and removed) from a bioreactor, typically expressed as some portion or multiple of the working volume, in a given time. “Working volume” refers to the amount of bioreactor volume used for cell culture. In one embodiment, the perfusion flow rate is one working volume or less per day. Perfusion feed medium can be formulated to maximize perfusion nutrient concentration to minimize perfusion rate.

[0190] Perfusion methods offer potential improvement over the batch and fed-batch methods by adding fresh media and simultaneously removing spent media. Typical large scale commercial cell culture strategies strive to reach high cell densities, $60 - 90(+) \times 10^6$ cells/mL where almost a third to over one-half of the reactor volume is biomass. With perfusion culture, extreme cell densities of $>1 \times 10^8$ cells/mL have been achieved and even higher densities are predicted. Typical perfusion cultures begin with a batch culture start-up lasting for a day or two followed by continuous, step-wise and/or intermittent addition of fresh feed media to the culture and simultaneous removal of spent media with the retention of cells and additional high molecular weight compounds such as proteins (based on the filter molecular weight cutoff) throughout the growth and production phases of the culture. Various methods, such as sedimentation, centrifugation, or filtration, can be used to remove spent media, while maintaining cell density. Perfusion flow rates of a fraction of a working volume per day up to many multiple working volumes per day have been reported.

[0191] An advantage of the perfusion process is that the production culture can be maintained for longer periods than batch or fed-batch culture methods. However, increased media preparation, use, storage and disposal are necessary to support a long-term perfusion culture, particularly those with high cell densities, which also need even more nutrients, and all of this drives the production costs even higher, compared to batch and fed batch methods. In addition, higher cell densities can cause problems during production, such as maintaining dissolved oxygen levels and problems with

increased gassing, including supplying more oxygen and removing more carbon dioxide, which would result in more foaming and the need for alterations to antifoam strategies; as well as during harvest and downstream processing where the efforts required to remove the excessive cell material can result in loss of product, negating the benefit of increased titer due to increased cell mass.

[0192] A large-scale cell culture strategy that combines fed batch feeding during the growth phase with continuous perfusion during the production phase may be used to express proteins of interest. Such a method may target a production phase in which the cell culture is maintained at a packed cell volume of less than or equal to 35%.

[0193] In one embodiment, a fed-batch culture with bolus feeds is used to maintain a cell culture during the growth phase. Perfusion feeding can then be used during a production phase. In one embodiment, perfusion begins when the cells have reached a production phase. In another embodiment, perfusion begins on or about day 3 to on or about day 9 of the cell culture. In another embodiment, perfusion begins on or about day 5 to on or about day 7 of the cell culture.

[0194] Using bolus feeding during the growth phase allows the cells to transition into the production phase, resulting in less dependence on a temperature shift as a means of initiating and controlling the production phase, however a temperature shift of about 36°C to about 31°C can take place between the growth phase and production phase. In one embodiment, the shift is from 36°C to 32°C.

[0195] In some embodiments, the bioreactor can be inoculated with at least 0.5×10^6 up to and beyond 3.0×10^6 viable cells/mL in a serum-free culture medium, for example, 1.0×10^6 viable cells/mL.

[0196] Cell cultures can be supplemented with concentrated feed medium containing components, such as nutrients and amino acids, which are consumed during the course of the production phase of the cell culture.

[0197] Concentrated feed medium may be based on just about any cell culture media formulation. Such a concentrated feed medium can contain most of the components of the cell culture medium at, for example, about 5X, 6X, 7X, 8X, 9X, 10X, 12X, 14X, 16X, 20X, 30X, 50X, 100X, 200X, 400X, 600X, 800X, or even about 1000X of their normal amount. Concentrated feed media are often used in fed batch culture processes.

[0198] Samples from the cell culture can be monitored and evaluated using any of the analytical techniques known in the art. A variety of parameters including recombinant protein and medium quality and characteristics can be monitored for the duration of the culture. Samples can be taken and monitored intermittently at a desirable frequency, including continuous monitoring, real time, or near real time.

[0199] Typically, the cell cultures that precede the final production culture (N-x to N-1) are used to generate the seed cells that will be used to inoculate the production bioreactor, the N-1 culture. The seed cell density can have a positive impact on the level of recombinant protein produced. Product

levels tend to increase with increasing seed density. Improvement in titer is tied not only to higher seed density but is likely to be influenced by the metabolic and cell cycle state of the cells that are placed into production.

[0200] Seed cells can be produced by any culture method. One such method is a perfusion culture using alternating tangential flow filtration. An N-1 bioreactor can be run using alternating tangential flow filtration to provide cells at high density to inoculate a production bioreactor. The N-1 stage may be used to grow cells to densities of $>90 \times 10^6$ cells/mL. The N-1 bioreactor can be used to generate bolus seed cultures or can be used as a rolling seed stock culture that could be maintained to seed multiple production bioreactors at high seed cell density. The duration of the growth stage of production can range from 7 to 14 days and can be designed so as to maintain cells in exponential growth prior to inoculation of the production bioreactor. Perfusion rates, medium formulation and timing are optimized to grow cells and deliver them to the production bioreactor in a state that is most conducive to optimizing their production. Seed cell densities of $>15 \times 10^6$ cells/mL can be achieved for seeding production bioreactors. Higher seed cell densities at inoculation can decrease or even eliminate the time needed to reach a desired production density.

[0201] In certain embodiments, the mammalian host cells can be used to generate high yield of a protein of interest. High yield, or high volumetric productivity, to the ability of cells to produce high levels of a protein of interest. The particular yield will depend on the protein of interest and can be at least 0.05 g/L, at least 0.1 g/L, at least 0.15 g/L, at least 0.2 g/L, at least 0.25 g/L, at least 0.3 g/L, at least 0.35 g/L, at least 0.4 g/L, at least 0.45 g/L, at least 0.5 g/L, at least 0.6 g/L, at least 0.7 g/L, at least 0.8 g/L, at least 0.9 g/L, at least 1 g/L, at least 1.5 g/L, at least 2 g/L, or more, in a 10-day culture grown in fed batch or perfusion conditions, using a feed medium suitable for the mammalian host cell and containing amino acids, vitamins, or trace elements. In specific embodiments, the host cells and methods of the present disclosure express a protein of interest and are capable of producing at least 0.5 g/L, at least 0.6 g/L, at least 0.7 g/L, at least 0.8 g/L, at least 0.9 g/L, at least 1 g/L, at least 1.5 g/L, at least 2 g/L, or more, preferably up to about 3 g/L, 4 g/L, 5 g/L or 10 g/L when grown under the culture conditions described above.

[0202] Yield can also be measured in terms of the specific productivity of a cell line, determined based on the amount of protein produced per cell per day (expressed as pg/cell/day). Mammalian host cells used with expression vector systems of the present disclosure are capable of producing at least 1 pg/cell/day, at least 2 pg/cell/day, at least 3 pg/cell/day, at least 4 pg/cell/day, at least 5 pg/cell/day, at least 6 pg/cell/day, at least 7 pg/cell/day, at least 8 pg/cell/day, at least 9 pg/cell/day, at least 10 pg/cell/day, at least 11 pg/cell/day, at least 12 pg/cell/day, at least 13 pg/cell/day, at least 14 pg/cell/day, at least 15 pg/cell/day, at least 20 pg/cell/day, at least 25 pg/cell/day, at least 50 pg/cell/day, at least 75 pg/cell/day or up to 100 pg/cell/day in a 10-day culture grown in fed batch or perfusion conditions, using a feed medium suitable for the mammalian host cell and containing amino

acids, vitamins, or trace elements. In specific embodiments, mammalian host cells used with expression vector systems of the present disclosure express an protein of interest and have a specific productivity of at least 10 pg/cell/day, at least 11 pg/cell/day, at least 12 pg/cell/day, at least 13 pg/cell/day, at least 14 pg/cell/day, at least 15 pg/cell/day, at least 20 pg/cell/day, at least 25 pg/cell/day, or more, preferably up to 50 pg/cell/day under the culture conditions described above.

[0203] The mammalian host cells described herein can be used to express a protein of interest. The expressed protein may be secreted into the culture medium from which they can be recovered and/or collected. In addition, the proteins can be purified, or partially purified, from such culture or component (e.g., from culture medium) using known processes and products available from commercial vendors. The purified proteins can then be “formulated”, meaning buffer exchanged, sterilized, bulk-packaged, and/or packaged for a final user. Suitable formulations for pharmaceutical compositions (i.e., pharmaceutically acceptable formulations) include those described in Remington’s Pharmaceutical Sciences, 18th ed. 1995, Mack Publishing Company, Easton, PA.

[0204] In certain embodiments, a CHO DHFR- cell or a CHO GSKO cell can be cultured under conditions to express antibody chains under methotrexate stringency in the case of a CHO DHFR- cell or methionine sulfoximine stringency in the case of a CHO GSKO cell to favor expression of the difficult-to-express chain paired with a stronger GS promoter.

[0205] In certain embodiments, a CHO DHFR- cell or a CHO GSKO cell can be cultured under conditions to express antibody chains under methotrexate stringency in the case of a CHO DHFR- cell or methionine sulfoximine stringency in the case of a CHO GSKO cell to favor expression of the difficult-to-express chain paired with a weaker GS promoter.

[0206] The present invention is not to be limited in scope by the specific embodiments described herein that are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

ADDITIONAL NON-LIMITING EXAMPLE EMBODIMENTS

[0207] Non-limiting example embodiments of the present disclosure also include:

E1. A method for preparing a multi-specific antibody having only one Fab or having two Fabs that are identical, wherein the method comprises the steps of:

- a) introducing two different expression vectors into a host cell, wherein the first expression vector encodes for 1) a light chain and 2) a heavy chain, heavy chain fusion, or Fc-fusion; and the second expression vector encodes for 1) the identical light chain, and 2) a heavy chain, heavy chain fusion or Fc-fusion; and
- b) inducing expression of said expression vectors to obtain said multi-specific antibody;

wherein said multi-specific antibody has only one Fab or has two Fabs that are identical.

E2. The method of E1, wherein one or both of the heavy chains or Fc regions are independently fused to a scFv, VH, or cytokine.

E3. The method of E1 or E2, wherein the multi-specific antibody is a bispecific antibody.

E4. The method of E1 or E2, wherein the multi-specific antibody is a trispecific antibody.

E5. The method of E3, wherein the bispecific antibody has an scFv, a VH, or a cytokine fused to either the N-terminus, the C-terminus, or between the CH1 and CH2 of one heavy chain either directly or through one or more linker sequences.

E6. The method of any of E1 to E5, wherein each of the expression vectors comprises a selectable marker which can be the same or different.

E7. The method of E6, wherein the selectable marker is selected from the group consisting of glutamine synthetase and dihydrofolate reductase.

E8. The method of E6 or E7, wherein a promoter operably linked to the nucleotide sequence encoding the selectable marker is selected from the group consisting of mPGK SR α , and SV40.

E9. The method of any one of E1 to E8, wherein the host cell is a mammalian host cell.

E10. The method of E9, wherein the mammalian host cell is a Chinese Hamster Ovary (CHO) cell.

E11. The method of E10, wherein the CHO cell is a dhfr- or GSKO.

E12. The method of any of E1 to E11, further comprising recovering the antibody from the culture.

E13. The method of E12, wherein the recovered antibody modality is purified and formulated in a pharmaceutically acceptable formulation.

[0208] Further non-limiting example embodiments/features of the present disclosure include:

F1. An expression system comprising:

- 1) a first expression vector which comprises a polynucleotide sequence which comprises the following elements in 5' to 3' order:
 - a) a first promoter operably linked to a nucleotide sequence encoding a light chain followed by a polyadenylation (polyA) signal sequence;
 - b) a second promoter operably linked to a nucleotide sequence encoding a heavy chain, a heavy chain fusion, or a Fc fusion followed by a second polyA signal sequence; and
 - c) a third promoter operably linked to a nucleotide sequence encoding a selectable marker followed by a third polyA signal sequence; and
- 2) a second expression vector which comprises a polynucleotide sequence which comprises the following elements in 5' to 3' order:
 - a) a first promoter operably linked to a nucleotide sequence encoding an identical copy of the light chain followed by a polyA signal sequence;
 - b) a second promoter operably linked to a nucleotide sequence encoding a heavy chain, a heavy chain fusion, or a Fc fusion followed by a second polyA signal sequence; and
 - c) a third promoter operably linked to a nucleotide sequence encoding a selectable marker followed by a third polyA signal sequence,

wherein, when expressed in a host cell, the expression system produces a multi-specific antibody that has only one Fab or has two Fabs that are identical.

F2. The expression system of F1, wherein each heavy chain fusion or Fc fusion is independently a fusion of a heavy chain or Fc with a VH, scFv, or cytokine fused to either the N-terminus or the C-terminus of the heavy chain portion of the heavy chain fusion or the Fc portion of the Fc fusion, or between the CH1 and CH2 of the heavy chain portion of the heavy chain fusion.

F3. The expression system of F1 or F2, wherein the first expression vector encodes a heavy chain, and the second expression vector encodes a heavy chain fusion having a VH, scFv, or cytokine fused to the C-terminus of the heavy chain portion of the heavy chain fusion.

F4. The expression system of F1 or F2, wherein the first expression vector encodes a heavy chain, and the second expression vector encodes a Fc fusion having a scFv fused to the N-terminus of the Fc portion of the Fc fusion.

F5. The expression system of F1 or F2, wherein the first expression vector encodes a heavy chain fusion having a VH or cytokine fused at the C-terminus of the heavy chain portion of the heavy chain

fusion, and the second expression vector encodes a heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the heavy chain fusion.

F6. An expression system comprising:

- 1) a first expression vector which comprises a polynucleotide sequence which comprises the following elements in 5' to 3' order:
 - a) a first promoter operably linked to a nucleotide sequence encoding a light chain followed by a polyadenylation (polyA) signal sequence;
 - b) a second promoter operably linked to a nucleotide sequence encoding a heavy chain followed by a second polyA signal sequence; and
 - c) a third promoter operably linked to a nucleotide sequence encoding a selectable marker followed by a third polyA signal sequence; and
- 2) a second expression vector which comprises a polynucleotide sequence which comprises the following elements in 5' to 3' order:
 - a) a first promoter operably linked to a nucleotide sequence encoding an identical copy of the light chain followed by a polyA signal sequence;
 - b) a second promoter operably linked to a nucleotide sequence encoding a heavy chain fusion followed by a second polyA signal sequence; and
 - c) a third promoter operably linked to a nucleotide sequence encoding a selectable marker followed by a third polyA signal sequence,

wherein, when expressed in a host cell, the expression system produces a multi-specific antibody that has two Fabs that are identical.

F7. The expression system of F6, wherein the heavy chain fusion is a fusion of a heavy chain with a VH, scFv, or cytokine.

F8. The expression system of F6 or F7, wherein the heavy chain fusion is a fusion of a heavy chain with a VH.

F9. The expression system of F6 or F7, wherein the heavy chain fusion is a fusion of a heavy chain with a scFv.

F10. The expression system of F6 or F7, wherein the heavy chain fusion is a fusion of a heavy chain with a cytokine.

F11. The expression system of F6 or F7, wherein the heavy chain fusion is a fusion of a heavy chain with a VH, scFv, or cytokine fused to either the N-terminus or the C-terminus of the heavy chain portion of the heavy chain fusion, or between the CH1 and CH2 of the heavy chain portion of the heavy chain fusion.

F12. The expression system of F6, F7, F8, or F11, wherein the heavy chain fusion is a fusion of a heavy chain with a VH fused to the N-terminus of the heavy chain portion of the heavy chain fusion.

F13. The expression system of F6, F7, F8, or F11, wherein the heavy chain fusion is a fusion of a heavy chain with a VH fused to the C-terminus of the heavy chain portion of the heavy chain fusion.

F14. The expression system of F6, F7, F8, or F11, wherein the heavy chain fusion is a fusion of a heavy chain with a VH fused between the CH1 and CH2 of the heavy chain portion of the heavy chain fusion.

F15. The expression system of F6, F7, F9, or F11, wherein the heavy chain fusion is a fusion of a heavy chain with a scFv fused to the N-terminus of the heavy chain portion of the heavy chain fusion.

F16. The expression system of F6, F7, F9, or F11, wherein the heavy chain fusion is a fusion of a heavy chain with a scFv fused to the C-terminus of the heavy chain portion of the heavy chain fusion.

F17. The expression system of F6, F7, F9, or F11, wherein the heavy chain fusion is a fusion of a heavy chain with a scFv fused between the CH1 and CH2 of the heavy chain portion of the heavy chain fusion.

F18. The expression system of F6, F7, F10, or F11, wherein the heavy chain fusion is a fusion of a heavy chain with a cytokine fused to the N-terminus of the heavy chain portion of the heavy chain fusion.

F19. The expression system of F6, F7, F10, or F11, wherein the heavy chain fusion is a fusion of a heavy chain with a cytokine fused to the C-terminus of the heavy chain portion of the heavy chain fusion.

F20. The expression system of F6, F7, F10, or F11, wherein the heavy chain fusion is a fusion of a heavy chain with a cytokine fused between the CH1 and CH2 of the heavy chain portion of the heavy chain fusion.

F21. An expression system comprising:

- 1) a first expression vector which comprises a polynucleotide sequence which comprises the following elements in 5' to 3' order:
 - a) a first promoter operably linked to a nucleotide sequence encoding a light chain followed by a polyadenylation (polyA) signal sequence;
 - b) a second promoter operably linked to a nucleotide sequence encoding a heavy chain followed by a second polyA signal sequence; and
 - c) a third promoter operably linked to a nucleotide sequence encoding a selectable marker followed by a third polyA signal sequence; and
- 2) a second expression vector which comprises a polynucleotide sequence which comprises the following elements in 5' to 3' order:
 - a) a first promoter operably linked to a nucleotide sequence encoding an identical copy of the light chain followed by a polyA signal sequence;
 - b) a second promoter operably linked to a nucleotide sequence encoding a Fc fusion followed by a second polyA signal sequence; and
 - c) a third promoter operably linked to a nucleotide sequence encoding a selectable marker followed by a third polyA signal sequence,

wherein, when expressed in a host cell, the expression system produces a multi-specific antibody that has only one Fab.

F22. The expression system of F21, wherein the Fc fusion is a fusion of a Fc with a VH, scFv, or cytokine.

F23. The expression system of F21 or F22, wherein the Fc fusion is a fusion of a Fc with a VH.

F24. The expression system of F21 or F22, wherein the Fc fusion is a fusion of a Fc with a scFv.

F25. The expression system of F21 or F22, wherein the Fc fusion is a fusion of a Fc with a cytokine.

F26. The expression system of F21 or F22, wherein the Fc fusion is a fusion of a Fc with a VH, scFv, or cytokine fused to either the N-terminus or the C-terminus of the Fc portion of the Fc fusion.

F27. The expression system of F21, F22, F23, or F26, wherein the Fc fusion is a fusion of a Fc with a VH fused to the N-terminus of the Fc portion of the Fc fusion.

F28. The expression system of F21, F22, F23, or F26, wherein the Fc fusion is a fusion of a Fc with a VH fused to the C-terminus of the Fc portion of the Fc fusion.

F29. The expression system of F21, F22, F24, or F26, wherein the Fc fusion is a fusion of a Fc with a scFv fused to the N-terminus of the Fc portion of the Fc fusion.

F30. The expression system of F21, F22, F24, or F26, wherein the Fc fusion is a fusion of a Fc with a scFv fused to the C-terminus of the Fc portion of the Fc fusion.

F31. The expression system of F21, F22, F25, or F26, wherein the Fc fusion is a fusion of a Fc with a cytokine fused to the N-terminus of the Fc portion of the Fc fusion.

F32. The expression system of F21, F22, F25, or F26, wherein the Fc fusion is a fusion of a Fc with a cytokine fused to the C-terminus of the Fc portion of the Fc fusion.

F33. An expression system comprising:

- 1) a first expression vector which comprises a polynucleotide sequence which comprises the following elements in 5' to 3' order:
 - a) a first promoter operably linked to a nucleotide sequence encoding a light chain followed by a polyadenylation (polyA) signal sequence;
 - b) a second promoter operably linked to a nucleotide sequence encoding a heavy chain fusion followed by a second polyA signal sequence; and
 - c) a third promoter operably linked to a nucleotide sequence encoding a selectable marker followed by a third polyA signal sequence; and
- 2) a second expression vector which comprises a polynucleotide sequence which comprises the following elements in 5' to 3' order:
 - a) a first promoter operably linked to a nucleotide sequence encoding an identical copy of the light chain followed by a polyA signal sequence;
 - b) a second promoter operably linked to a nucleotide sequence encoding a heavy chain fusion followed by a second polyA signal sequence; and
 - c) a third promoter operably linked to a nucleotide sequence encoding a selectable marker followed by a third polyA signal sequence,

wherein, when expressed in a host cell, the expression system produces a multi-specific antibody that has two Fabs that are identical.

F34. The expression system of F33, wherein each heavy chain fusion is independently a fusion of a heavy chain with a VH, scFv, or cytokine.

F35. The expression system of F33 or F34, wherein each heavy chain fusion is independently a fusion of a heavy chain with a VH, scFv, or cytokine fused to either the N-terminus or the C-terminus of the heavy chain portion of the heavy chain fusion, or between the CH1 and CH2 of the heavy chain portion of the heavy chain fusion.

F36. The expression system of F33, F34, or F35, wherein the first expression vector encodes a heavy chain fusion having a VH, scFv, or cytokine fused at the C-terminus of the heavy chain portion of the heavy chain fusion, and the second expression vector encodes a heavy chain fusion having a VH, scFv, or cytokine fused at the N-terminus of the heavy chain portion of the heavy chain fusion.

F37. The expression system of any one of F33-F36, wherein the first expression vector encodes a heavy chain fusion having a VH or cytokine fused at the C-terminus of the heavy chain portion of the heavy chain fusion, and the second expression vector encodes a heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the heavy chain fusion.

F38. The expression system of any one of F33-F37, wherein the first expression vector encodes a heavy chain fusion having a VH fused at the C-terminus of the heavy chain portion of the heavy chain fusion, and the second expression vector encodes a heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the heavy chain fusion.

F39. The expression system of any one of F33-F37, wherein the first expression vector encodes a heavy chain fusion having a cytokine fused at the C-terminus of the heavy chain portion of the heavy chain fusion, and the second expression vector encodes a heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the heavy chain fusion.

F40. The expression system of any one of F1-F39, wherein the first expression vector and the second expression vector are both mammalian expression vectors.

F41. The expression system of any one of F1-F40, wherein the selectable marker on the first expression vector and the selectable marker on the second expression vector are independently glutamine synthetase or dihydrofolate reductase.

- F42. The expression system of any one of F1-F41, where the selectable marker on the first expression vector and the selectable marker on the second expression vector are both glutamine synthetase.
- F43. The expression system of any one of F1-F41, wherein the selectable marker on the first expression vector and the selectable marker on the second expression vector are both dihydrofolate reductase.
- F44. The expression system of any one of F1-F43, wherein each of the first, second, and third polyA signal sequences on the first expression vector and the first, second, and third polyA signal sequences on the second expression vector is independently selected from the group consisting of a bovine growth hormone (BGH) polyA signal sequence, a thymidine kinase polyA (TKpA) signal sequence, a rabbit beta-globin polyA signal sequence, and a simian virus 40 (SV40) early polyA signal sequence.
- F45. The expression system of any one of F1-F44, wherein the first, second, and third polyA signal sequences on the first expression vector are the same.
- F46. The expression system of any one of F1-F44, wherein the first, second, and third polyA signal sequences on the first expression vector are different.
- F47. The expression system of any one of F1-F46, wherein the first, second, and third polyA signal sequences on the first expression vector are the same.
- F48. The expression system of any one of F1-F46, wherein the first, second, and third polyA signal sequences on the first expression vector are different.
- F49. The expression system of any one of F1-F43, F45, or F47, wherein each of the first, second, and third polyA signal sequences on the first expression vector and the first, second, and third polyA signal sequences on the second expression vector are the same.
- F50. The expression system of any one of F1-F43, F45, F47, or F49, wherein the first, second, and third polyA signal sequences on the first expression vector and the first, second, and third polyA signal sequences on the second expression vector are each a simian virus 40 (SV40) early polyA signal sequence.

- F51. The expression system of any one of F1-F43, F45, F47, or F49, wherein:
each of the first, second, and third polyA signal sequences on the first expression vector and the first, second, and third polyA signal sequences on the second expression vector are the same; and
the selectable marker on the first expression vector and the selectable marker on the second expression vector are both glutamine synthetase.
- F52. The expression system of any one of F1-F43, F45, F47, or F49-F51, wherein:
the first, second, and third polyA signal sequences on the first expression vector and the first, second, and third polyA signal sequences on the second expression vector are each a simian virus 40 (SV40) early polyA signal sequence; and
the selectable marker on the first expression vector and the selectable marker on the second expression vector are both glutamine synthetase.
- F53. The expression system of any one of F1-F52, wherein the third promoter on the first expression vector and the third promoter on the second expression vector are independently selected from the group consisting of mPGK, SR α , and SV40 promoters.
- F54. The expression system of any one of F1-F53, wherein the third promoter on the first expression vector and the third promoter on the second expression vector are the same.
- F55. The expression system of any one of F1-F54, wherein the third promoter on the first expression vector and the third promoter on the second expression vector are both mPGK promoters.
- F56. The expression system of any one of F1-F54, wherein the third promoter on the first expression vector and the third promoter on the second expression vector are both SR α promoters.
- F57. The expression system of any one of F1-F54, wherein the third promoter on the first expression vector and the third promoter on the second expression vector are both SV40 promoters.
- F58. The expression system of any one of F1-F53, wherein the third promoter on the first expression vector and the third promoter on the second expression vector are different.
- F59. The expression system of any one of F1-F40, wherein:
each of the first, second, and third polyA signal sequences on the first expression vector and the first, second, and third polyA signal sequences on the second expression vector is independently selected from the group consisting of a bovine growth hormone (BGH) polyA signal sequence, a

thymidine kinase polyA (TKpA) signal sequence, a rabbit beta-globin polyA signal sequence, and a simian virus 40 (SV40) early polyA signal sequence;

the selectable marker on the first expression vector and the selectable marker on the second expression vector are independently glutamine synthetase or dihydrofolate reductase; and

the third promoter on the first expression vector and the third promoter on the second expression vector are independently selected from the group consisting of mPGK, SR α , and SV40 promoters.

F60. The expression system of any one of F1-F40 or F59, wherein:

each of the first, second, and third polyA signal sequences on the first expression vector and the first, second, and third polyA signal sequences on the second expression vector is the same, wherein the polyA signal sequence is selected from the group consisting of a bovine growth hormone (BGH) polyA signal sequence, a thymidine kinase polyA (TKpA) signal sequence, a rabbit beta-globin polyA signal sequence, and a simian virus 40 (SV40) early polyA signal sequence;

the selectable marker on the first expression vector and the selectable marker on the second expression vector are both glutamine synthetase; and

the third promoter on the first expression vector and the third promoter on the second expression vector are the same, wherein the third promoter is selected from the group consisting of mPGK, SR α , and SV40 promoters.

F61. The expression system of any one of F1-F40, F59, or F60, wherein:

each of the first, second, and third polyA signal sequences on the first expression vector and the first, second, and third polyA signal sequences on the second expression vector is a simian virus 40 (SV40) early polyA signal sequence;

the selectable marker on the first expression vector and the selectable marker on the second expression vector are both glutamine synthetase; and

the third promoter on the first expression vector and the third promoter on the second expression vector are both SR α promoters.

F62. The expression system of any one of F1-F61, wherein at least one of the first promoter on the first expression vector, the first promoter on the second expression vector, the second promoter on the first expression vector, and the second promoter on the second expression vector is a GAPDH promoter.

F63. The expression system of any one of F1-F62, wherein each of the first promoter on the first expression vector, the first promoter on the second expression vector, the second promoter on the first expression vector, and the second promoter on the second expression vector is a GAPDH promoter.

F64. The expression system of any one of F1-F63, wherein at least one of the first promoter on the first expression vector, the first promoter on the second expression vector, the second promoter on the first expression vector, and the second promoter on the second expression vector is a CMV/GAPDH promoter.

F65. The expression system of any one of F1-F64, wherein each of the first promoter on the first expression vector, the first promoter on the second expression vector, the second promoter on the first expression vector, and the second promoter on the second expression vector is a CMV/GAPDH promoter.

F66. The expression system of any one of F1-F40, wherein:
each of the first promoter on the first expression vector, the first promoter on the second expression vector, the second promoter on the first expression vector, and the second promoter on the second expression vector is a GAPDH promoter;
each of the first, second, and third polyA signal sequences on the first expression vector and the first, second, and third polyA signal sequences on the second expression vector is the same, wherein the polyA signal sequence is selected from the group consisting of a bovine growth hormone (BGH) polyA signal sequence, a thymidine kinase polyA (TKpA) signal sequence, a rabbit beta-globin polyA signal sequence, and a simian virus 40 (SV40) early polyA signal sequence;
the selectable marker on the first expression vector and the selectable marker on the second expression vector are both glutamine synthetase; and
the third promoter on the first expression vector and the third promoter on the second expression vector are the same, wherein the third promoter is selected from the group consisting of mPGK, SR α , and SV40 promoters.

F67. The expression system of any one of F1-F40, wherein:
each of the first promoter on the first expression vector, the first promoter on the second expression vector, the second promoter on the first expression vector, and the second promoter on the second expression vector is a GAPDH promoter;
each of the first, second, and third polyA signal sequences on the first expression vector and the first, second, and third polyA signal sequences on the second expression vector is a simian virus 40 (SV40) early polyA signal sequence;

the selectable marker on the first expression vector and the selectable marker on the second expression vector are both glutamine synthetase; and

the third promoter on the first expression vector and the third promoter on the second expression vector are both SR α promoters.

F68. The expression system of any one of F1-F40, wherein:

each of the first promoter on the first expression vector, the first promoter on the second expression vector, the second promoter on the first expression vector, and the second promoter on the second expression vector is a CMV/GAPDH promoter;

each of the first, second, and third polyA signal sequences on the first expression vector and the first, second, and third polyA signal sequences on the second expression vector is the same, wherein the polyA signal sequence is selected from the group consisting of a bovine growth hormone (BGH) polyA signal sequence, a thymidine kinase polyA (TKpA) signal sequence, a rabbit beta-globin polyA signal sequence, and a simian virus 40 (SV40) early polyA signal sequence;

the selectable marker on the first expression vector and the selectable marker on the second expression vector are both glutamine synthetase; and

the third promoter on the first expression vector and the third promoter on the second expression vector are the same, wherein the third promoter is selected from the group consisting of mPGK, SR α , and SV40 promoters.

F69. The expression system of any one of F1-F40, wherein:

each of the first promoter on the first expression vector, the first promoter on the second expression vector, the second promoter on the first expression vector, and the second promoter on the second expression vector is a CMV/GAPDH promoter;

each of the first, second, and third polyA signal sequences on the first expression vector and the first, second, and third polyA signal sequences on the second expression vector is a simian virus 40 (SV40) early polyA signal sequence;

the selectable marker on the first expression vector and the selectable marker on the second expression vector are both glutamine synthetase; and

the third promoter on the first expression vector and the third promoter on the second expression vector are both SR α promoters.

F70. A mammalian host cell comprising an expression system of any one of F1-F69.

F71. The mammalian host cell of F70, which is a Chinese hamster ovary (CHO) cell.

F72. The mammalian host cell of F71, wherein the CHO cell is a dihydrofolate reductase deficient (DHFR-) CHO cell or a glutamine synthetase knockout (GSKO) CHO cell.

F73. The mammalian host cell of F71 or F72, wherein the CHO cell is a dihydrofolate reductase deficient (DHFR-) CHO cell.

F74. The mammalian host cell of F71 or F72, wherein the CHO cell is a glutamine synthetase knockout (GSKO) CHO cell.

F75. A method for producing a multi-specific antibody having only one Fab or having two Fabs that are identical, wherein the method comprises:

- a) introducing an expression system of any one of F1-F69 into a mammalian host cell; and
- b) culturing the mammalian host cell to produce the multi-specific antibody.

F76. The method of F75, further comprising isolating the multi-specific antibody.

F77. A method for producing a multi-specific antibody having only one Fab or having two Fabs that are identical, wherein the method comprises the steps of:

- a) culturing a mammalian host cell of any one of F70-F74 to produce the multi-specific antibody; and
- b) isolating the multi-specific antibody.

F78. The method of F76 or F77, further comprising purifying the multi-specific antibody and formulating it in a pharmaceutically acceptable formulation.

F79. A method for preparing a multi-specific antibody having only one Fab or having two Fabs that are identical, wherein the method comprises:

- a) introducing two different expression vectors into a host cell, wherein the first expression vector encodes for 1) a light chain, and 2) a heavy chain, a heavy chain fusion, or a Fc fusion; and the second expression vector encodes for 1) the identical light chain, and 2) a heavy chain, a heavy chain fusion, or a Fc fusion; and
- b) culturing the host cell in a mammalian cell culture to express the expression vectors to produce the multi-specific antibody, wherein the multi-specific antibody has only one Fab or has two Fabs that are identical.

F80. The method of F79, wherein each of the heavy chain fusions or Fc fusions are a heavy chain or a Fc independently fused to a scFv, a VH, or a cytokine.

F81. The method of F79 or F80, wherein the multi-specific antibody is a bispecific antibody.

F82. The method of F81, wherein the bispecific antibody has 1) a heavy chain; and 2) a heavy chain fusion or Fc fusion with a scFv, a VH, or a cytokine fused to either the N-terminus or the C-terminus of the heavy chain portion of the heavy chain fusion or the Fc portion of the Fc fusion, or between the CH1 and CH2 of the heavy chain portion of the heavy chain fusion.

F83. The method of F79 or F80, wherein the multi-specific antibody is a trispecific antibody.

F84. The method of F83, wherein the trispecific antibody has 1) a first heavy chain fusion having a VH or a cytokine fused to either the N-terminus or the C-terminus of the heavy chain portion of the first heavy chain fusion; and 2) a second heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the second heavy chain fusion.

F85. The method of any of F79 to F84, wherein each of the expression vectors comprises a selectable marker, which can be the same or different.

F86. The method of F85, wherein each selectable marker is selected from the group consisting of glutamine synthetase and dihydrofolate reductase.

F87. The method of F85 or F86, wherein each of the expression vectors further comprises a promoter operably linked to the nucleotide sequence encoding the selectable marker, wherein each promoter is independently selected from the group consisting of mPGK, SR α , and SV40 promoters.

F88. The method of any one of F79-F87, wherein the host cell is a mammalian host cell.

F89. The method of F88, wherein the mammalian host cell is a Chinese Hamster Ovary (CHO) cell.

F90. The method of F89, wherein the CHO cell is a dihydrofolate reductase deficient (dhfr-) CHO cell or a glutamine synthetase knockout (GSKO) CHO cell.

F91. The method of any one of F79-F90, further comprising recovering the multi-specific antibody from the culture.

F92. The method of F91, wherein the recovered antibody modality is purified and formulated in a pharmaceutically acceptable formulation.

EXAMPLES

EXAMPLE 1. Vector Engineering Strategy to Improve Productivity for a C1mAb

Summary

[0209] Establishing stable Chinese Hamster Ovary (CHO) cell lines producing therapeutic recombinant antibodies involves integration of the heavy and light chains (HC and LC, respectively) from an expression vector(s) into the genome through a selection process. In the glutamine synthetase knockout (GS KO) expression system, selection of stable pools can be controlled by balancing the expression level of the exogenous GS gene and the concentration of its specific inhibitor, L-methionine sulfoximine (MSX).

[0210] This Example describes a further vector system optimization by using a 2xLC strategy with a three-chain C1mAb antibody to modulate the expression levels of the HC1, HC2-scFv and common LC, and the GS genes in combination with various MSX concentrations to improve productivity in CHO cells. The vector engineering strategy described herein can be expanded to improve and optimize productivity for other recombinant proteins expressed in stable pools in general.

Materials and Methods

[0211] *Plasmid Generation.* The coding sequences of the LC, HC1, and HC2-ScFv were inserted in the pPBGS plasmid backbone using Golden Gate cloning to generate multi-cistronic vectors. Briefly, CMV/GAPDH promoters/enhancer fragments were used to control the LCs and HCs with SV40-Poly A fragments following the SR α promoters driving the expression of mGS-polyA in that order. The vector configurations are shown in FIG. 2B. All the fragments were uni-directionally assembled using combinations of overhang sequences to facilitate Golden Gate cloning. MSX was used at 0 and 12.5 μ M.

[0212] *Transfection of Plasmids into GS KO Host.* A GS KO (knockout) clonal cell host, derived from the CHO-K1 parental host, was used for generating stable pools expressing the C1mAb. Host cells were passaged at a seeding density of $0.4-0.3 \times 10^6$ cells/mL every 3-4 days in a proprietary DMEM-F12-based media in shake flasks at 120 rpm, 36°C, and 5% CO₂. Twenty-four hours before transfection, the host cells were seeded at 1×10^6 cells/mL to ensure the cells would be in the exponential growth phase at transfection.

[0213] Stable pools expressing the antibodies were generated using a Gene Pulser XCell (BioRad Laboratories; Hercules, CA) following the manufacturer's protocol. Duplicate transfections were performed for each of the vector configurations. Briefly, 20 µg of each plasmid in combination with 5 µg of a proprietary piggybac transposase were electroporated into 20×10^6 host cells. The transfected cells were recovered in 20 mL of growth media in 50 mL spin tubes at 225 rpm, 36°C, and 5% CO₂.

[0214] *Selection and Recovery.* Seventy-two hours post transfection, the cells were spun down and transferred into selection media without glutamine or growth factor and with 0 and 25 µM of MSX. The cells were passaged at seeding densities around $1-2 \times 10^6$ cells/mL every 3-4 days until viability reached over 90%, when the seeding density was reduced to $0.4-0.3 \times 10^6$ cells/mL.

[0215] *Fed-batch Production.* Fully recovered cells were inoculated for fed-batch production at 1×10^6 cells/mL in a proprietary basal media. The cultures were supplemented with proprietary feeds on days 3, 6, and 8 and harvested on day 10. Cell count and viability were determined using a Vi-Cell BLU cell viability analyzer (Beckman Coulter, Brea, CA). Supernatants were analyzed for titer (protein A-HPLC).

Results

[0216] Results in FIGs. 3A-3B show higher titers (A) and cell specific productivity (B) obtained using the 2xLC strategy at different MSX levels.

[0217] Additionally, FIGs. 4A-4B show higher effective titer (A) and higher nrCE-SDS-Main Peak (B) for vector configurations during fed-batch production. Color corresponds to the MSX concentration levels.

EXAMPLE 2. Vector Engineering Strategy to Improve Productivity for a AmAb Bispecific Molecule Summary

[0218] Establishing stable Chinese Hamster Ovary (CHO) cell lines producing therapeutic recombinant antibodies and molecules derived from antibodies involves integration of the heavy and light chains (HC and LC, respectively) from an expression vector(s) into the genome through a selection process. In the glutamine synthetase knockout (GS KO) expression system, selection of stable pools can be controlled by balancing the expression level of the exogenous GS gene and the concentration of its specific inhibitor, L-methionine sulfoximine (MSX).

[0219] This Example describes a further vector optimization by using promoters with different strengths to modulate the expression levels of the HC, LC, and GS genes in combination with MSX to improve productivity of AmAb bispecific antibodies in CHO cells. The vector engineering strategy described herein can be expanded to improve and optimize productivity for other recombinant proteins expressed in stable pools in general.

Materials and Methods

[0220] *AmAb Plasmid Generation.* The coding sequences of a LC, HC1, and scFv-Fc were inserted in the pPBGS plasmid backbone using Golden Gate cloning to generate multi-cistronic vectors. Briefly, CMV/GAPDH promoters/enhancer fragments were used to control the HC, scFv-Fc, and LC with SV40-Poly A fragments following the SR α promoter driving the expression of mGS-polyA in that order. The vector configurations are shown in FIG. 5. All the fragments were uni-directionally assembled using combinations of overhang sequences to facilitate Golden Gate cloning. MSX was used at 25 μ M.

[0221] *Transfection of Plasmids into GS KO Host.* A GS KO (knockout) clonal cell host, derived from the CHO-K1 parental host, was used for generating stable pools expressing the AmAb. Host cells were passaged at a seeding density of $0.3\text{--}0.4 \times 10^6$ cells/mL every 3-4 days in a proprietary media in shake flasks at 130 rpm, 36°C, and 5% CO₂. Twenty-four hours before transfection, the host cells were seeded at 1×10^6 cells/mL to ensure the cells would be in exponential growth phase at transfection.

[0222] Stable pools expressing the AmAb were generated using a Lipofectamine LTX (Gibco, Billings, MT) and Opti-MEM I Reduced Serum Media (Gibco, Billings, MT) following the manufacturer's protocol. Single transfection was performed for each of the vector configurations. Briefly, 2 μ g of each plasmid in combination with 2 μ g of a proprietary piggybac transposase were added into 4×10^6 host cells. The transfected cells were recovered in 4 mL of growth media in a 6-well plate at 225 rpm, 36°C, and 5% CO₂.

[0223] *Selection and Recovery.* Seventy-two hours post transfection, the cells were spun down and transferred into selection media without glutamine and with 25 μ M of MSX. The cells were passaged at seeding densities around $1\text{--}2 \times 10^6$ cells/mL every 3-4 days until viability reached over 90%, when the seeding density was reduced to $0.3\text{--}0.4 \times 10^6$ cells/mL.

[0224] *Fed-batch Production.* Fully recovered cells were inoculated for fed-batch production at 1×10^6 cells/mL in a proprietary basal media. The cultures were supplemented with proprietary feeds on days 3, 6, and 8 and harvested on day 10. Cell count and viability were determined using a Vi-Cell BLU cell viability analyzer (Beckman Coulter, Brea, CA). Supernatants were analyzed for 1) titer (protein A-HPLC) and 2) product quality attributes including aggregates, clips, and isoforms using size-exclusion chromatography (SEC-UHPLC) (Waters UPLC H-class series), reduced capillary electrophoresis (rCE-SDS) (Sciex PA 800 Plus Pharmaceutical Analysis System), and analytical hydrophobic interaction chromatography (HIC-HPLC) (Agilent HPLC 1100/1200 series), respectively.

Results

[0225] Results in FIGs. 6A-6B show higher normalized yield (A) and % nrMCE MP (B) obtained using the 2xLC configuration compared to the 1xLC configurations.

EXAMPLE 3. Vector Engineering Strategy to Improve Productivity for a Trispecific Molecule

Summary

[0226] Establishing stable Chinese Hamster Ovary (CHO) cell lines producing therapeutic recombinant antibodies and molecules derived from antibodies involves integration of the heavy and light chains (HC and LC, respectively) from an expression vector(s) into the genome through a selection process. In the glutamine synthetase knockout (GS KO) expression system, selection of stable pools can be controlled by balancing the expression level of the exogenous GS gene and the concentration of its specific inhibitor, L-methionine sulfoximine (MSX).

[0227] This Example describes a further vector optimization by using promoters with different strengths to modulate the expression levels of the HC, LC, and GS genes in combination with various MSX concentrations to improve productivity of trispecific antibodies in CHO cells. The vector engineering strategy described herein can be expanded to improve and optimize productivity for other recombinant proteins expressed in stable pools in general.

Materials and Methods

[0228] *Trispecific Plasmid Generation.* The coding sequences of the LCs and HCs were inserted in the pPBGS plasmid backbone using Golden Gate cloning to generate multi-cistronic vectors. Briefly, the CMV/GAPDH promoter/enhancer fragment were used to control the LCs and HCs with SV40-Poly A fragments following the SR α promoters driving the expression of mGS-polyA in that order. The vector configurations are shown in FIG. 7. All the fragments were uni-directionally assembled using combinations of overhang sequences to facilitate Golden Gate cloning. MSX was used at 25 μ M.

[0229] *Transfection of Plasmids into GS KO Host.* A GS KO (knockout) clonal cell host, derived from the CHO-K1 parental host, was used for generating stable pools expressing the trispecific antibody. Host cells were passaged at a seeding density of $0.3\text{--}0.4 \times 10^6$ cells/mL every 3-4 days in a proprietary based media in shake flasks at 130 rpm, 36°C and 5% CO₂. Twenty-four hours before transfection, the host cells were seeded at 1×10^6 cells/mL to ensure the cells would be in exponential growth phase at transfection.

[0230] Stable pools expressing the trispecific antibody were generated using a Lipofectamine LTX (Gibco, Billings, MT) and Opti-MEM I Reduced Serum Media (Gibco, Billings, MT) following the manufacturer's protocol. Single transfection was performed for each of the vector configurations. Briefly, 2 μ g of each plasmid in combination with 2 μ g of a proprietary piggybac transposase were added into 4×10^6 host cells. The transfected cells were recovered in 4 mL of growth media in a 6-well plate at 225 rpm, 36°C and 5% CO₂.

[0231] *Selection and Recovery.* Seventy-two hours post transfection, the cells were spun down and transferred into selection media without glutamine and with 25 μ M of MSX. The cells were passaged at seeding densities around $1-2 \times 10^6$ cells/mL every 3-4 days until viability reached over 90%, when the seeding density was reduced to $0.3-0.4 \times 10^6$ cells/mL.

[0232] *Fed-batch Production.* Fully recovered cells were inoculated for fed-batch production at 1×10^6 cells/mL in a proprietary basal media. The cultures were supplemented with proprietary feeds on days 3, 6, and 8 and harvested on day 10. Cell count and viability were determined using a Vi-Cell BLU cell viability analyzer (Beckman Coulter, Brea, CA). Supernatants were analyzed for 1) titer (protein A-HPLC) and 2) product quality attributes including aggregates, clips, and isoforms using size-exclusion chromatography (SEC-UHPLC) (Waters UPLC H-class series), reduced capillary electrophoresis (rCE-SDS) (Sciex PA 800 Plus Pharmaceutical Analysis System), and analytical hydrophobic interaction chromatography (HIC-HPLC) (Agilent HPLC 1100/1200 series), respectively.

Results

[0233] Results in FIGs. 8A-8B show higher normalized yield (A) and % nrMCE MP (B) for the 2xLC configuration compared to the 1xLC configuration.

What is claimed is:

1. An expression system comprising:
 - 1) a first expression vector which comprises a polynucleotide sequence which comprises the following elements in 5' to 3' order:
 - a) a first promoter operably linked to a nucleotide sequence encoding a light chain followed by a polyadenylation (polyA) signal sequence;
 - b) a second promoter operably linked to a nucleotide sequence encoding a heavy chain, a heavy chain fusion, or a Fc fusion followed by a second polyA signal sequence; and
 - c) a third promoter operably linked to a nucleotide sequence encoding a selectable marker followed by a third polyA signal sequence; and
 - 2) a second expression vector which comprises a polynucleotide sequence which comprises the following elements in 5' to 3' order:
 - a) a first promoter operably linked to a nucleotide sequence encoding an identical copy of the light chain followed by a polyA signal sequence;
 - b) a second promoter operably linked to a nucleotide sequence encoding a heavy chain, a heavy chain fusion, or a Fc fusion followed by a second polyA signal sequence; and
 - c) a third promoter operably linked to a nucleotide sequence encoding a selectable marker followed by a third polyA signal sequence,wherein, when expressed in a host cell, the expression system produces a multi-specific antibody that has only one Fab or has two Fabs that are identical.
2. The expression system of claim 1, wherein each heavy chain fusion or Fc fusion is independently a fusion of a heavy chain or Fc with a VH, scFv, or cytokine fused to either the N-terminus or the C-terminus of the heavy chain portion of the heavy chain fusion or the Fc portion of the Fc fusion, or between the CH1 and CH2 of the heavy chain portion of the heavy chain fusion.
3. The expression system of claim 1 or claim 2, wherein the first expression vector encodes a heavy chain, and the second expression vector encodes a heavy chain fusion having a VH, scFv, or cytokine fused to the C-terminus of the heavy chain portion of the heavy chain fusion.
4. The expression system of claim 1 or claim 2, wherein the first expression vector encodes a heavy chain, and the second expression vector encodes a Fc fusion having a scFv fused to the N-terminus of the Fc portion of the Fc fusion.

5. The expression system of claim 1 or claim 2, wherein the first expression vector encodes a heavy chain fusion having a VH or cytokine fused at the C-terminus of the heavy chain portion of the heavy chain fusion, and the second expression vector encodes a heavy chain fusion having a scFv fused at the N-terminus of the heavy chain portion of the heavy chain fusion.
6. The expression system of any one of claims 1-5, wherein the first expression vector and the second expression vector are both mammalian expression vectors.
7. The expression system of any one of claims 1-6, wherein the selectable marker on the first expression vector and the selectable marker on the second expression vector are independently glutamine synthetase or dihydrofolate reductase.
8. The expression system of any one of claims 1-7, where the selectable marker on the first expression vector and the selectable marker on the second expression vector are both glutamine synthetase.
9. The expression system of any one of claims 1-8, wherein each of the first, second, and third polyA signal sequences on the first expression vector and the first, second, and third polyA signal sequences on the second expression vector is independently selected from the group consisting of a bovine growth hormone (BGH) polyA signal sequence, a thymidine kinase polyA (TKpA) signal sequence, a rabbit beta-globin polyA signal sequence, and a simian virus 40 (SV40) early polyA signal sequence.
10. The expression system of any one of claims 1-9, wherein the first, second, and third polyA signal sequences on the first expression vector and the first, second, and third polyA signal sequences on the second expression vector are the same.
11. The expression system of any one of claims 1-10, wherein each of the first, second, and third polyA signal sequences on the first expression vector and the first, second, and third polyA signal sequences on the second expression vector is a simian virus 40 (SV40) early polyA signal sequence.
12. The expression system of any one of claims 1-11, wherein:
each of the first, second, and third polyA signal sequences on the first expression vector and the first, second, and third polyA signal sequences on the second expression vector is a simian virus 40 (SV40) early polyA signal sequence; and

the selectable marker on the first expression vector and the selectable marker on the second expression vector are both glutamine synthetase.

13. The expression system of any one of claims 1-12, wherein the third promoter on the first expression vector and the third promoter on the second expression vector are independently selected from the group consisting of mPGK, SR α , and SV40 promoters.

14. The expression system of any one of claims 1-13, wherein the third promoter on the first expression vector and the third promoter on the second expression vector are the same.

15. The expression system of any one of claims 1-14, wherein the third promoter on the first expression vector and the third promoter on the second expression vector are both SR α promoters.

16. The expression system of any one of claims 1-15, wherein each of the first promoter on the first expression vector, the first promoter on the second expression vector, the second promoter on the first expression vector, and the second promoter on the second expression vector is a GAPDH promoter.

17. The expression system of any one of claims 1-16, wherein each of the first promoter on the first expression vector, the first promoter on the second expression vector, the second promoter on the first expression vector, and the second promoter on the second expression vector is a CMV/GAPDH promoter.

18. A mammalian host cell comprising an expression system of any one of claims 1-17.

19. The mammalian host cell of claim 18, which is a Chinese hamster ovary (CHO) cell.

20. The mammalian host cell of claim 18 or claim 19, wherein the CHO cell is a glutamine synthetase knockout (GSKO) CHO cell.

21. A method for producing a multi-specific antibody having only one Fab or having two Fabs that are identical, wherein the method comprises:

- a) introducing an expression system of any one of claims 1-17 into a mammalian host cell;
and
- b) culturing the mammalian host cell to produce the multi-specific antibody.