with non-stochastic evolution of the antibody/protein inside of an optimized mammalian host cell, increases the likelihood of success and greatly accelerates the process for generating an optimized antibody/protein that will express at high enough levels in mammalian cells desired in manufacturing.

SUMMARY

[0005] The present disclosure provides methods of integrating therapeutic protein (including antibodies) generation and/or selection, evolution and expression in a eukaryotic host, such as a mammalian cell host or a yeast cell host, for manufacturing in a single system. Therapeutic proteins, including antibodies, are generated, optimized and manufactured in the same eukaryotic host system. The disclosed system of Comprehensive Integrated Antibody Optimization (CIAO!™) allows for simultaneous evolution of protein performance and expression optimization.

[0005a] In one aspect, the present disclosure refers to a method of evolution of a template human protein in a manufacturing host that is a eukaryotic cell production host, the method comprising: a. evolving the template human protein to produce a set of mutant human proteins in the eukaryotic cell production host; b. screening the set of mutant human proteins for at least one predetermined property, characteristic or activity; c. selecting an up-mutant human protein from the set of mutant human proteins based upon the at least one predetermined property, characteristic or activity; and d. manufacturing the up-mutant human protein comprising expressing the up-mutant human protein in the same eukaryotic cell production host as used in the evolving step (a).

[0006] In one example the disclosure provides a method of selection, evolution and expression of an antibody in a mammalian cell production host; the method comprising generating an anti-antigen antibody library in a mammalian cell production host with antibody cell surface display; screening the library for at least one predetermined property, characteristic or activity; selecting a template antibody from the library; evolving the template antibody to produce a set of mutant antibodies in the mammalian cell production host with antibody cell surface display;

screening the mutant antibodies for the at least one predetermined property, characteristic or activity; selecting an up-mutant antibody from the set of mutant antibodies based upon optimization of the at least one predetermined property, characteristic or activity when compared to the template antibody; and expressing the up-mutant antibody in the same mammalian cell production host as used in the generating step. In one example, the antigen is pre-selected. In another example, the anti-antigen antibody library is a humanized anti-antigen antibody library.

[0007] In one example, the mammalian cell production host is selected from 3T3 mouse fibroblast cells; BHK21 Syrian hamster fibroblast cells; MDCK, dog epithelial cells; Hela human epithelial cells; PtK1 rat kangaroo epithelial cells; SP2/0 mouse plasma cells; and NS0 mouse mouse plasma cells; HEK 293 human embryonic kidney cells; COS monkey kidney cells; CHO, CHO-S Chinese hamster ovary cells; R1 mouse embryonic cells; E14.1 mouse embryonic cells; H1 human embryonic cells; H9 human embryonic cells; PER C.6, human embryonic cells; S. cerevisiae yeast cells; or picchia yeast cells. In a particular example, the mammalian system is CHO-S or HEK293. In one example, the screening steps utilize fluorescence-activated cell sorting (FACS).

[0008] In another example, the evolving step comprises producing a set of mutant antibodies formed from the template antibody having m complementary determining regions (CDR), wherein m is an integer selected from 1, 2, 3, 4, 5 or 6, each said CDR comprising n amino acid residues, the method comprising generating m x n separate sets of antibodies, each set comprising member antibodies having X number of different predetermined amino acid residues at a single predetermined position of the CDR; wherein each set of antibodies differs in the single predetermined position; and the number of different member antibodies generated is equivalent to m x n x X. In a particular example, m is 6.

[0009] In one example, the evolving step comprises generating n-1 separate sets of mutant polypeptides from the template antibody, each set comprising member polypeptides having X number of different predetermined amino acid residues at a single predetermined position of the polypeptide; wherein each set of polypeptides differs in the single predetermined position; and the number of different member polypeptides generated is equivalent to [n-1] x X. In one example, X represents the

19 naturally occurring amino acid residues not present in a given position of the template polypeptide.

[0010] In another example, the screening step comprises assaying each member polypeptide for at least one predetermined property, characteristic or activity; identifying any change in said property, characteristic or activity of the member polypeptide relative to the template polypeptide; creating a functional map wherein the functional map is used to identify positions and mutations in the mutant polypeptide which result in an up-mutant and/or a silent mutation compared to the template polypeptide.

[0011] In another example, the functional map is used to identify one or more of the group consisting of (a) positions and mutations which do not affect the activity of the mutant polypeptide compared to the template polypeptide; (b) fully mutable sites compared to the template polypeptide; and (c) positions and mutations which result in an up-mutant compared to the template polypeptide.

[0012] In another example, the antibody fragment is selected from a heavy chain, light chain, variable domain, constant domain, hypervariable region, complementarity determining region 1 (CDR1), complementarity determining region 2 (CDR2), and complementarity determining region 3 (CDR3).

[0013] In another example, the generating step comprises subjecting a codon-containing polynucleotide encoding for said template polypeptide to polymerase-based amplification using a 64-fold degenerate oligonucleotide for each codon to be mutagenized, wherein each of said 64-fold degenerate oligonucleotides is comprised of a first homologous sequence and a degenerate N,N,N triplet sequence, so as to generate a set of progeny polynucleotides; and subjecting said set of progeny polynucleotides to clonal amplification such that polypeptides encoded by the progeny polynucleotides are expressed.

[0014] In another example, the disclosure refers to the method as described wherein the predetermined property, characteristic or activity is selected from reduction of protein-protein aggregation, enhancement of protein stability, increased protein solubility, introduction of glycosylation sites, introduction of conjugation sites, reduction of immunogenicity, enhancement of protein expression, increase in

antigen affinity, decrease in antigen affinity, change in binding affinity, change in immunogenicity, or enhancement of specificity.

[0015] In another example, the disclosure provides a method of evolution and expression of an antibody in a mammalian cell production host; the method comprising selecting a template antibody; evolving the template antibody to produce a set of mutant antibodies in a mammalian cell production host with antibody cell surface display; screening the mutant antibodies for the at least one predetermined property, characteristic or activity; selecting an up-mutant antibody from the set of mutant antibodies based upon optimization of the at least one predetermined property, characteristic or activity compared to the template antibody; and expressing the up-mutant antibody in the same mammalian cell production host used in the evolving step.

[0016] In one example, the screening step comprises creating a functional map wherein the functional map is used to identify positions and mutations in the mutant polypeptide which result in an up-mutant and/or a silent mutation compared to the template polypeptide. In another example, the screening steps comprise fluorescence-activated cell sorting (FACS).

[0017] In one example, the mammalian cell production host is selected from 3T3 mouse fibroblast cells; BHK21 Syrian hamster fibroblast cells; MDCK, dog epithelial cells; Hela human epithelial cells; PtK1 rat kangaroo epithelial cells; SP2/0 mouse plasma cells; and NS0 mouse mouse plasma cells; HEK 293 human embryonic kidney cells; COS monkey kidney cells; CHO, CHO-S Chinese hamster ovary cells; R1 mouse embryonic cells; E14.1 mouse embryonic cells; H1 human embryonic cells; H9 human embryonic cells; and PER C.6, human embryonic cells. In a particular example, the mammalian system is CHO-S or HEK293. In another example, the cell production host is selected from CHOK1SV or NSO host cell lines.

[0018] In another example, the disclosure provides a method of evolution and expression of a protein in a eukaryotic cell production host; the method comprising selecting a template antibody; evolving the template antibody to produce a set of mutant antibodies in a eukaryotic cell production host; screening the mutant antibodies for at least one predetermined property, characteristic or activity;

selecting an up-mutant antibody from the set of mutant antibodies based upon optimization of the at least one predetermined property, characteristic or activity compared to the template antibody; and expressing the up-mutant antibody in the same eukaryotic cell production host as in the evolving step for any commercial scale.

[0019] In one example, the disclosure provides a method of evolution and manufacturing of a human protein in a cell production host; the method comprising generating a human protein library in a cell production host selected from one of the group consisting of a bacterial or eukaryotic production host; screening the library for at least one predetermined property, characteristic or activity; selecting a template human protein from the library based upon the at least one predetermined property, characteristic or activity; evolving the template human protein to produce a set of mutant human proteins in the cell production host; screening the set of mutant human proteins for the at least one predetermined property, characteristic or activity and screening for modified expression; selecting an up-mutant human protein from the set of mutant human proteins based upon (1) optimization of the at least one predetermined property, characteristic or activity and (2) modified expression when compared to the template human protein; and manufacturing the human protein comprising expressing the up-mutant human protein in the same production host as in the generating step. In one example, the modified expression in the selecting step is improved expression.

[0020] In another example, the evolving step comprises an evolution technique selected from one of comprehensive positional evolution (CPE); comprehensive positional insertion evolution (CPI); comprehensive positional deletion evolution (CPD); comprehensive positional evolution (CPE) followed by combinatorial protein synthesis (CPS); comprehensive positional deletion evolution (CPD) followed by combinatorial protein synthesis (CPS); or comprehensive positional deletion evolution (CPD) followed by combinatorial protein synthesis (CPS).

[0021] In one example, the human protein is an antibody. In another example, the antibody is a full length antibody.

[0022] In another example, the at least one predetermined property, characteristic or activity in screening step (e) comprises one or more of (1) screening

for a silent mutation and (2) screening for a missense mutation; compared to the template antibody.

[0023] In another example, one or more portions of the antibody selected from Fc and Fv; framework; and one or more CDRs are modified in the up-mutant human antibody compared to the template human antibody is evolved.

[0024] In another example, the screening step comprises screening the set of mutant human proteins for the at least one predetermined property, characteristic or activity and screening for modified expression simultaneously.

[0025] In a further example, the human protein for evolution and manufacturing is selected from an enzyme cytokine, receptor, DNA binding protein, chelating agent or hormone.

[0026] In another example, the cell production host is a eukaryotic production host and the evolving step comprises evolving the template human protein to produce a set of mutant human proteins in the eukaryotic cell production host with cell surface display.

[0027] In another example, the disclosure provides a method of evolution for enhanced expression and manufacturing of a human protein in a eukaryotic cell production host; the method comprising selecting a template human protein for evolution; evolving the template human protein comprising generation of mutant codons encoding the template human protein to produce a set of mutant human proteins in the production host; screening the set of mutant human proteins for at least one predetermined property, characteristic or activity and screening for enhanced expression when compared to the template human protein; selecting an up-mutant human protein from the set of mutant human proteins based upon (1) retention or optimization of the at least one predetermined property, characteristic or activity and (2) enhanced expression when compared to the template human protein; and manufacturing the up-mutant human protein comprising expressing the up-mutant human protein in the same production host as in the evolving step.

[0028] In one example, the mutant codons of the up-mutant human protein result in at least one silent mutation and/or missense mutation. In another example, the mutant codons of the up-mutant human protein result in at least one silent mutation.

[0029] In a further example, the template human protein is an approved ethical protein therapeutic drug, and the up-mutant human protein is a biosimilar.

[0030] In another example, the selecting step comprises selecting an up-mutant human protein from the set of mutant human proteins based upon (1) optimization of the at least one predetermined property, characteristic or activity and (2) enhanced expression when compared to the template human protein.

[0031] In another example, the disclosure provides a method of identifying and producing a target human protein, the method comprising generating an human protein library in a eukaryotic cell production host with protein cell surface display; screening the library for at least one predetermined property, characteristic or activity; identifying a target human protein from the library based upon the at least one predetermined property, characteristic or activity; and expressing the target human protein in the same eukaryotic cell production host as in the generating step to produce a target human protein. In one example, the target human protein is an antibody. In another example, the antibody is a full length antibody.

[0032] In another example, the disclosure provides a method of evolution of a human protein in a manufacturing host, the method comprising mutating a template human protein to produce a set of mutant human proteins in a manufacturing host; and screening the set of mutant progeny proteins for at least one predetermined property, characteristic or activity. In one example, the method further comprises selecting an up-mutant human protein from the set of mutant human proteins based upon the at least one predetermined property, characteristic or activity. In another example, the method further comprises manufacturing the up-mutant human protein comprising expressing the up-mutant human protein in the same production host as in the mutating step. In another example, the selecting step further comprises selecting an up-mutant human protein from the set of mutant human proteins based upon (1) optimization of the at least one predetermined property, characteristic or activity when compared to the template human protein, and (2) modified expression when compared to the template human protein. In one example, the modified expression is enhanced expression.

[0033] In another example, the disclosure provides a method of evolution and manufacturing of a human protein in a cell manufacturing host; the method

comprising mutating a template human protein to produce a set of mutant human proteins in a manufacturing host; screening the set of mutant human proteins for at least one predetermined property, characteristic or activity and screening for modified expression;

selecting an up-mutant human protein from the set of mutant human proteins based upon (1) optimization of the at least one predetermined property, characteristic or activity, and (2) modified expression when compared to the template human protein; and manufacturing the human protein comprising expressing the up-mutant human protein in the same manufacturing host as in the mutating step.

[0034] In a further example, the disclosure provides a method of evolution for enhanced expression and manufacturing of a human protein in a eukaryotic cell production host; the method comprising mutating a template human protein comprising generation of mutant codons encoding the template human protein to produce a set of mutant human proteins in a manufacturing host; screening the set of mutant human proteins for at least one predetermined property, characteristic or activity and screening for enhanced expression when compared to the template human protein; selecting an up-mutant human protein from the set of mutant human proteins based upon (1) retention or optimization of the at least one predetermined property, characteristic or activity and (2) enhanced expression when compared to the template human protein; and manufacturing the up-mutant human protein in the same manufacturing host as in the mutating step.

[0035] In one example, the screening step comprises screening the set of mutant human proteins for the at least one predetermined property, characteristic or activity and screening for enhanced expression simultaneously.

BRIEF DESCRIPTION OF THE DRAWINGS

[0036] Figure 1 provides a schematic of the CIAO!™ method of integrating therapeutic protein (e.g., antibody) generation and/or selection, evolution and expression in a eukaryotic host, such as a mammalian cell host or a yeast cell host, for manufacturing in a single system.

[0037] Figure 2 illustrates how comprehensive positional evolution (CPE^{TM}) is used to generate a molecule specific database (EvoMap^{TM}).

[0038] Figure 3 shows a schematic of Comprehensive Positional Synthesis (CPS™) which can be used to combine up-mutants from CPE™.

[0039] Figure 4 shows a schematic of Comprehensive Positional Insertion (CPI™) evolution.

[0040] Figure 5 illustrates one combination of evolution methods: a lengthened nucleic acid from CPI™ evolution is subjected to Comprehensive Positional Evolution (CPE™) and used to generate a molecule specific database (EvoMap™).

[0041] Figure 6 shows a schematic of Comprehensive Positional Deletion (CPD™) evolution.

[0042] Figure 7 illustrates another combination of evolution methods: a shortened nucleic acid from CPD™ evolution is subjected to Comprehensive

WE CLAIM:

1. A method of evolution of a template human protein in a manufacturing host that is a eukaryotic cell production host, the method comprising:
    a. evolving the template human protein to produce a set of mutant human proteins in the eukaryotic cell production host;
    b. screening the set of mutant human proteins for at least one predetermined property, characteristic or activity;
    c. selecting an up-mutant human protein from the set of mutant human proteins based upon the at least one predetermined property, characteristic or activity; and
    d. manufacturing the up-mutant human protein comprising expressing the up-mutant human protein in the same eukaryotic cell production host as used in the evolving step (a).

2. The method of claim 1, wherein the template human protein is selected by:
    a1. generating a human protein library in a cell production host selected from one of the group consisting of a bacterial and a eukaryotic cell production host;
    a2. screening the library for the at least one predetermined property, characteristic or activity; and
    a3. selecting the template human protein from the library based upon the at least one predetermined property, characteristic or activity, wherein
    the selecting step (c) comprises:
    selecting the up-mutant human protein from the set of mutant human proteins based upon (1) optimization of the at least one predetermined property, characteristic or activity and (2) modified expression when compared to the template human protein.

3. The method of claim 2, wherein the modified expression is improved expression.

4. The method of claim 1 wherein the evolving step (a) comprises one of comprehensive positional evolution (CPE); comprehensive positional insertion evolution (CPI); comprehensive positional deletion evolution (CPD); comprehensive positional evolution (CPE) followed by combinatorial protein synthesis (CPS); comprehensive

positional deletion evolution (CPD) followed by combinatorial protein synthesis (CPS); or comprehensive positional deletion evolution (CPD) followed by combinatorial protein synthesis (CPS).

5. The method of claim 1 wherein the template human protein is a template antibody and the at least one predetermined property, characteristic or activity in screening step (b) comprises one or more of (1) a silent mutation and (2) a missense mutation, compared to the template antibody.

6. The method of claim 1 wherein the template human protein is selected from an enzyme, a cytokine, a receptor, a DNA binding protein, a chelating agent, and a hormone.

7. The method of claim 1 wherein the evolving step (a) comprises evolving the template human protein to produce the set of mutant human proteins in the eukaryotic cell production host with cell surface display.

8. The method of claim 1, wherein the screening step (b) comprises:

screening the set of mutant human proteins for the at least one predetermined property, characteristic or activity and screening for enhanced expression when compared to the template human protein; and

the selecting step (c) comprises:

selecting the up-mutant human protein from the set of mutant human proteins based upon (1) retention or optimization of the at least one predetermined property, characteristic or activity and (2) enhanced expression when compared to the template human protein.

9. The method of claim 8 wherein the template human protein is an approved protein therapeutic drug and the up-mutant human protein is a biosimilar.

10. The method of claim 8, wherein the selecting step (c) comprises selecting the up-mutant human protein from the set of mutant human proteins based upon (1) optimization of the at least one predetermined property, characteristic or activity and (2) enhanced expression when compared to the template human protein.

11. The method of claim 1 wherein the selecting step (c) further comprises

selecting the up-mutant human protein from the set of mutant human proteins based upon (1) optimization of the at least one predetermined property, characteristic or activity when compared to the template human protein, and (2) modified expression when compared to the template human protein.

12. The method of claim 11 wherein the modified expression is enhanced expression.

13. The method of claim 1, wherein the screening step (b) comprises:

screening the set of mutant human proteins for the at least one predetermined property, characteristic or activity and screening for enhanced expression when compared to the template human protein; and

the selecting step (c) comprises:

selecting the up-mutant human protein from the set of mutant human proteins based upon (1) retention or optimization of the at least one predetermined property, characteristic or activity and (2) enhanced expression when compared to the template human protein.

14. The method of claim 13 wherein the screening step (b) comprises screening the set of mutant human proteins for the at least one predetermined property, characteristic or activity and screening for enhanced expression simultaneously.

15. The method of claim 2, wherein the cell production host in step (a1) is the same eukaryotic cell production host used in steps (a) and (d).