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(54) **Title:** METHODS FOR MODULATING PROTEIN GLYCOSYLATION PROFILES OF RECOMBINANT PROTEIN THERAPEUTICS USING MONOSACCHARIDES AND OLIGOSACCHARIDES

(57) **Abstract:** The present invention relates to the field of protein production, and in particular to methods and compositions for modulating glycosylation of proteins expressed in host cells.



METHODS FOR MODULATING PROTEIN GLYCOSYLATION PROFILES OF RECOMBINANT PROTEIN THERAPEUTICS USING MONOSACCHARIDES AND OLIGOSACCHARIDES

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RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application Serial No. 61/785,901, filed on March 14, 2013, the entire contents of which are incorporated herein by reference.

10

BACKGROUND OF THE INVENTION

The instant invention relates to the field of protein (*e.g.*, antibody or DVD-Ig) production, and, in particular, to methods and compositions for controlling and limiting the heterogeneity of proteins expressed in host cells. The production of proteins for
15 biopharmaceutical applications typically involves the use of cell cultures that are known to produce proteins exhibiting varying levels of heterogeneity. The basis for such heterogeneity includes, but is not limited to, the presence of distinct glycosylation profiles in the produced proteins. For example, but not by way of limitation, such heterogeneity can be observed as an increase in high mannose N-glycans and NGA2F-GlcNAc species as well as a decrease in
20 fucosylated species, such as NGA2F species.

The glycosylation profile of a protein (*e.g.*, an antibody or DVD-Ig) can influence its biological activity through changes in half-life due to effects on clearance, folding, stability and antibody-dependent cellular cytotoxicity (ADCC) (Shental-Behor D. *et al.*, (2008) PNAS 105:8256-8261; Kuhlmann M. *et al.*, (2006) Nephrol. Dial. Transplant 21:v4-v8;
25 Zheng K. *et al.*, (2011) mAbs 3(6):568-576). ADCC is one mechanism responsible for the therapeutic effect of antibodies such as the anti-CD20 IgG1 rituximab and the anti-Her2/neu IgG1 trastuzumab. ADCC activity is influenced by the amount of fucose linked to the innermost GlcNAc of the Fc region, with enhanced activity seen with a reduction in fucose (Mori K. *et al.*, (2007) Cytotechnology 55:109-114).

30 Heterogeneity of protein glycosylation can be assayed by releasing oligosaccharides present on the protein of interest (*e.g.*, an antibody or DVD-Ig) via enzymatic digestion with, for example, N-glycanase. Once the glycans are released, the free reducing end of each glycan can be labeled by reductive amination with a fluorescent tag. The resulting labeled

glycans are separated by normal-phase HPLC (NP-HPLC) and detected by a fluorescence detector for quantitation. Technological advances in recombinant protein production analysis have provided unique opportunities for identifying the extent of glycosylation exhibited by a particular protein population, particularly in the context of large-scale production of recombinant proteins.

Although such advances have allowed for the robust characterization of protein glycosylation, there remains a need in the art for culture conditions and production methods that allow for control over the glycosylation profile of a protein therapeutic. Modulation of protein glycosylation is particularly advantageous in the context of cell culture processes used for commercially produced recombinant bio-therapeutics as glycosylation has the potential to impact therapeutic utility. Control of the glycosylation profile of a therapeutic protein (*e.g.*, an antibody or an antigen binding fragment thereof, or a DVD-Ig) is also critical for ensuring the production of comparable proteins such as biosimilars. Accordingly, there is a need in the art for compositions and methods for the targeted modulation of protein glycosylation. The instant invention addresses this need by providing compositions and methods for modulating protein glycosylation. The invention further provides methods for the targeted modulation of mannosylated and fucosylated N-glycan species linked to a protein of interest (*e.g.*, antibody or DVD-Ig).

SUMMARY OF THE INVENTION

In one aspect, the present invention provides methods of producing a composition comprising a protein with a modulated glycosylation profile. The methods include culturing a host cell expressing the protein in cell culture media supplemented with a monosaccharide and/or an oligosaccharide, thereby producing the composition comprising the protein with a modulated glycosylation profile as compared to a control, wherein the control is a composition comprising the protein produced by culturing a host cell expressing the protein in the same cell culture media but which is not supplemented with a monosaccharide and/or an oligosaccharide.

In one embodiment, the methods further comprise purifying the composition comprising the protein with a modulated glycosylation profile.

In another embodiment, the protein is an antibody or antigen-binding portion thereof. In a particular embodiment, the antibody is an anti-TNF α antibody. In yet another

embodiment the anti-TNF α antibody is adalimumab, or an antigen binding fragment thereof. In yet another embodiment, the protein is a dual variable domain immunoglobulin (DVD-Ig). In one embodiment, the protein is selected from the group consisting of a TVD-Ig, a half-body and a RAB.

5 In one embodiment of the invention, the monosaccharide is tagatose. In another embodiment, the oligosaccharide is sucrose.

In one embodiment, the cell culture media is supplemented with a sufficient amount of the monosaccharide, *e.g.*, tagatose, to achieve a monosaccharide concentration selected from the group consisting of about 1mM, 10 mM, 30 mM, 50 mM and 70 mM. In a particular
10 embodiment, the monosaccharide, *e.g.*, tagatose, concentration is 30 mM.

In one embodiment, the cell culture media is supplemented with a sufficient amount of the oligosaccharide, *e.g.*, sucrose, to achieve an oligosaccharide concentration selected from the group consisting of about 1 mM, 7 mM, 10 mM, 15 mM, 30 mM, 50 mM and 70 mM. In a particular embodiment, the oligosaccharide, *e.g.*, sucrose, concentration is 30 mM.

15 In another embodiment, the modulated glycosylation profile of the protein comprises modulation of a fucosylation level and/or a mannosylated N-glycan oligosaccharide level in the protein.

In another embodiment, the modulation of the fucosylation level comprises a decrease in the fucosylation level in the protein, *e.g.*, a decrease in the level of NGA2F, NA1F-
20 GlcNAc, NA1F and/or NA2F in the protein. In a further embodiment, the decrease in the level of NGA2F, NA1F-GlcNAc, NA1F and/or NA2F is a decrease of about 0.1%, 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60% or 65%.

In one embodiment, the modulation of the fucosylation level comprises an increase in
25 the fucosylation level in the protein, *e.g.*, an increase in the level of NGA2F-GlcNAc, NA1F-GlcNAc, NA1F and/or NA2F in the protein. In another embodiment, the increase in the level of NGA2F-GlcNAc, NA1F-GlcNAc, NA1F or NA2F is an increase of about 0.1%, 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15% or 20%.

In one embodiment, an overall decrease in the fucosylation level comprises an
30 increase or a decrease in the level of NGA2F, NGA2F-GlcNAc, NA1F-GlcNAc, NA1F and/or NA2F in the protein. In another embodiment, the decrease in the fucosylation level is a decrease of about 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45% or 50%.

In another embodiment, the modulation of the mannosylated N-glycan level comprises an increase in the mannosylation level of the protein. In one embodiment, the mannosylation level is an increase of about 0.1%, 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45% or 50%. In yet another embodiment, the increase in the mannosylation level comprises an increase in the level of a high mannose N-glycan oligosaccharide selected from the group consisting of Man 5 glycan, Man 6 glycan, Man 7 glycan and Man 8 glycan. In one embodiment, the levels of Man 5 glycan, Man 6 glycan, Man 7 glycan and/or Man 8 glycan are increased by about 0.1%, 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40, 45% or 50%.

In one embodiment of the present invention the host cell is a CHO cell.

In another aspect, the present invention provides methods of producing compositions comprising an antibody, or antigen binding fragment thereof, with a modulated glycosylation profile. The methods include culturing a host cell expressing the antibody, or antigen binding fragment thereof, in cell culture media supplemented with sucrose and/or tagatose, thereby producing the composition comprising the antibody, or antigen binding fragment thereof, with an increased level of mannosylated N-glycans and a decreased level of fucosylated N-glycans as compared to a control, wherein the control is a composition comprising an antibody, or antigen binding fragment thereof, produced by culturing a host cell expressing the antibody, or antigen binding fragment thereof, in cell culture media which is not supplemented with tagatose and/or glucose. In one embodiment, the antibody is adalimumab, or an antigen binding fragment thereof.

In another aspect, the present invention provides methods of producing compositions comprising an antibody, or antigen binding fragment thereof, with a modulated glycosylation profile. The methods include culturing a host cell expressing the antibody, or antigen binding fragment thereof, in cell culture media supplemented with sucrose and/or tagatose, thereby producing the composition comprising the antibody, or antigen binding fragment thereof, with a 1-50% increase in the level of mannosylated N-glycans and a 1-50% decrease in the level of fucosylated N-glycans as compared to a control, wherein the control is a composition comprising an antibody, or antigen binding fragment thereof, produced by culturing a host cell expressing the antibody, or antigen binding fragment thereof, in cell culture media which is not supplemented with tagatose and/or glucose. In one embodiment, the antibody is adalimumab, or an antigen binding fragment thereof.

In a further aspect, the present invention provides compositions comprising a cell culture media comprising a monosaccharide and/or an oligosaccharide. In one embodiment, the monosaccharide is tagatose. In another embodiment, the oligosaccharide is sucrose.

5 In yet another aspect, the present invention provides pharmaceutical compositions comprising the protein compositions produced by the methods of the invention and a pharmaceutically acceptable carrier.

In another aspect, the present invention provides compositions comprising a therapeutic protein with a modulated glycosylation profile produced by the methods the invention. In one embodiment, the therapeutic protein is an antibody.

10 In another aspect, the present invention provides compositions comprising a therapeutic protein, wherein the protein comprises a 1-50% increase in the level of mannosylated N-glycans and a 1-50% decrease in the level of fucosylated N-glycans, as compared to the control, wherein the control is a composition comprising a protein produced by culturing a host cell expressing the protein in cell culture media which is not
15 supplemented with a monosaccharide and/or an oligosaccharide. In one embodiment, the therapeutic protein is selected from the group consisting of an antibody, an antigen-binding portion thereof, DVD-Ig, TVD-Ig, RAB and half-body. In one embodiment, the therapeutic protein is an antibody.

In another aspect, the present invention provides methods of producing compositions
20 comprising an antibody, or antigen binding fragment thereof, with a modulated glycosylation profile by culturing a host cell expressing the antibody, or antigen binding fragment thereof, in cell culture media supplemented with sucrose and/or tagatose, thereby producing the composition comprising the antibody, or antigen binding fragment thereof, with a 1-30% increase in antibody-dependent cellular cytotoxicity (ADCC) response as compared to a
25 control, wherein the control is a composition comprising an antibody, or antigen binding fragment thereof, produced by culturing a host cell expressing the antibody, or antigen binding fragment thereof, in cell culture media which is not supplemented with tagatose and/or glucose. In one embodiment the antibody is adalimumab, or an antigen binding fragment thereof.

30

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the chemical structure of sucrose, tagatose and fructose.

Figure 2 depicts a simplified linear reaction view of the N-glycan biosynthetic pathway in mammalian cells.

5 **Figures 3A-3C** depict the cell culture performance of Cell Line 1 in media supplemented with 30 mM, 50 mM or 70 mM sucrose. Figure 3A: Viable cell density. Figure 3B: Percent viability. Figure 3C: Harvest titer ratio. Control is unsupplemented media.

10 **Figure 4** depicts the N-glycan oligosaccharide results as an absolute percent change in oligosaccharide profile of Cell Line 1 in media supplemented with 30 mM, 50 mM or 70 mM sucrose.

15 **Figures 5A-5C** depict the cell culture performance of Cell Line 1 in media supplemented with 30 mM, 50 mM or 70 mM tagatose. Figure 5A: Viable cell density. Figure 5B: Percent viability. Figure 5C: Harvest titer ratio. Control is unsupplemented media.

Figure 6 depicts the N-glycan oligosaccharide results as an absolute percent change in oligosaccharide profile of Cell Line 1 in media supplemented with 30 mM, 50 mM or 70 mM tagatose.

20 **Figures 7A-7F** depict the cell culture performance of Cell Line 1 in laboratory-scale bioreactors with media supplemented with 50 mM sucrose. Figure 7A: Viable cell density. Figure 7B: pCO₂. Figure 7C: Osmolality. Figure 7D: Percent viability. Figure 7E: Lactate. Figure 7F: Harvest titer ratio. Control is unsupplemented media.

25 **Figure 8** depicts the N-glycan oligosaccharide results as an absolute percent change in oligosaccharide profile of Cell Line 1 in laboratory-scale bioreactors with media supplemented with 50 mM sucrose.

Figures 9A-9F depict the cell culture performance of Cell Line 1 in laboratory-scale bioreactors with media supplemented with 50 mM tagatose. Figure 9A: Viable cell density. Figure 9B: pCO₂. Figure 9C: Osmolality. Figure 9D: Percent viability. Figure 9E: Lactate. Figure 9F: Harvest titer ratio. Control is unsupplemented media.

Figure 10 depicts the N-glycan oligosaccharide results as an absolute percent change in oligosaccharide profile of Cell Line 1 in laboratory-scale bioreactors with media supplemented with 50 mM tagatose.

Figures 11A-11C depict the cell culture performance of Cell Line 2 in media supplemented with 7 mM, 15 mM or 30 mM sucrose. Figure 11A: Viable cell density. Figure 11B: Percent viability. Figure 11C: Harvest titer ratio. Control is unsupplemented media.

Figure 12 depicts the N-glycan oligosaccharide results as an absolute percent change in oligosaccharide profile of Cell Line 2 in media supplemented with 7 mM, 15 mM or 30 mM sucrose.

Figures 13A-13D depict the cell culture performance of Cell Line 1 in media supplemented with 1 mM, 10 mM, 30 mM, 50 mM or 70 mM sucrose. Figure 13A: Viable cell density. Figure 13B: Percent viability. Figure 13C: Relative harvest titer compared to unsupplemented control. Figure 13D: Absolute % change in protein oligosaccharide profile compared to unsupplemented control. (*p < 0.05 on marked day or process condition indicating a statistically significant difference compared to the unsupplemented control).

Figures 14A-14D depict the cell culture performance of Cell Line 1 in media supplemented with 1 mM, 10 mM, 30 mM, 50 mM or 70 mM tagatose. Figure 14A: Viable cell density. Figure 14B: Percent viability. Figure 14C: Relative harvest titer compared to unsupplemented control. Figure 14D: Absolute % change in protein oligosaccharide profile compared to unsupplemented control. (*p < 0.05 on marked day or process condition indicating a statistically significant difference compared to the unsupplemented control).

Figures 15A-15D depict the cell culture performance of Cell Line 2 in media supplemented with 1 mM, 30 mM, or 50 mM sucrose. Figure 15A: Viable cell density. Figure 15B: Percent viability. Figure 15C: Relative harvest titer compared to unsupplemented control. Figure 15D: Absolute % change in protein oligosaccharide profile compared to unsupplemented control. (*p < 0.05 on marked day or process condition indicating a statistically significant difference compared to the unsupplemented control).

Figures 16A-16D depict the cell culture performance of Cell Line 2 in media supplemented with 1 mM, 30 mM, or 50 mM tagatose. Figure 16A: Viable cell density. Figure 16B: Percent viability. Figure 16C: Relative harvest titer compared to unsupplemented control. Figure 16D: Absolute % change in protein oligosaccharide profile

compared to unsupplemented control. (* $p < 0.05$ on marked day or process condition indicating a statistically significant difference compared to the unsupplemented control).

Figures 17A-17F depict the cell culture performance of Cell Line 1 in laboratory-scale bioreactors with media supplemented with 50 mM sucrose, tagatose, or fructose. Figure 17A: Viable cell density. Figure 17B: Percent viability. Figure 17C: Glucose concentration. Figure 17D: Lactate concentration. Figure 17E: Osmolality. Figure 17F: Relative harvest titer compared to unsupplemented control.

Figure 18 depicts the absolute percent change in protein oligosaccharide profile of Cell Line 1 in laboratory-scale bioreactors with media supplemented with 50 mM sucrose, tagatose, or fructose.

Figure 19 is a schematic representation of various protein therapeutics (*e.g.*, antibody, DVD-Ig, TVD-Ig, RAB, Half-body) whose glycosylation profiles may be modulated using the methods of the present invention.

Figure 20 depicts the absolute percent change in protein oligosaccharide profile of Cell Line 3 in shake flask cultures with media supplemented with 7 mM, 15 mM or 50 mM sucrose.

Figure 21 depicts the Cr-51 release assay results for the measurement of antibody dependent cell cytotoxicity (ADCC) for antibodies purified from host cell cultures supplemented with 7 mM, 15 mM or 50 mM sucrose. The control was an unsupplemented culture.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods and compositions for modulating the glycosylation profile of a protein such as a therapeutic protein (*e.g.*, antibody, DVD-Ig, TVD-Ig, Half-body or RAB compositions).

The present invention is based on the identification and optimization of upstream process technologies, *e.g.*, recombinant cell culture conditions, for protein production, *e.g.*, production of antibodies or antigen-binding portions thereof or DVD-Igs, resulting in the production of protein compositions with modulated glycosylation profiles (*e.g.*, decreased fucosylation and/or increased mannosylation).

I. Definitions

In order that the present invention may be more readily understood, certain terms are first defined.

Unless otherwise defined herein, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. The meaning and scope of the terms should be clear, however, in the event of any latent ambiguity, definitions provided herein take precedent over any dictionary or extrinsic definition. Further, unless otherwise required by context, singular terms, for example, those characterized by “a” or “an”, shall include pluralities, *e.g.*, one or more impurities. In this application, the use of “or” means “and/or”, unless stated otherwise. Furthermore, the use of the term “including,” as well as other forms of the term, such as “includes” and “included”, is not limiting. Also, terms such as “element” or “component” encompass both elements and components comprising one unit and elements and components that comprise more than one unit unless specifically stated otherwise.

Most naturally occurring peptides (or proteins) comprise carbohydrate or saccharide moieties attached to the peptide *via* specific linkages to a select number of amino acids along the length of the primary peptide chain. Thus, many naturally occurring peptides are termed “glycopeptides” or “glycoproteins” or are referred to as “glycosylated” proteins or peptides.

The term “glycoform” refers to an isoform of a protein, *e.g.*, an antibody, that differs only with respect to the number and/or type of attached glycan(s). Glycoproteins often consist of a number of different glycoforms.

The predominant sugars found on glycoproteins are glucose, galactose, mannose, fucose, N-acetylgalactosamine (“GalNAc”), N-acetylglucosamine (“GlcNAc”) and sialic acid (*e.g.*, N-acetylneuraminic acid (“NANA” or “NeuAc”, where “Neu” is neuraminic acid) and “Ac” refers to “acetyl”). The processing of the sugar groups occurs co-translationally in the lumen of the ER and continues in the Golgi apparatus for N-linked glycoproteins.

The oligosaccharide structure attached to the peptide chain is known as a “glycan” molecule. The glycan structures found in naturally occurring glycopeptides are typically divided into two classes, “N-linked glycans” or N-linked oligosaccharides” and “O-linked glycans” or O-linked oligosaccharides”.

Peptides expressed in eukaryotic cells typically comprise N-glycans. “N-glycans” are N-glycosylated at an amide nitrogen of an asparagine or an arginine residue in a protein *via*

an N-acetylglucosamine residue. These “N-linked glycosylation sites” occur in the peptide primary structure containing, for example, the amino acid sequence asparagine-X-serine/threonine, where X is any amino acid residue except proline and aspartic acid.

Techniques for the determination of glycan primary structure are well known in the art and are described in detail, for example, in Montreuil, "Structure and Biosynthesis of Glycopeptides" In *Polysaccharides in Medicinal Applications*, pp. 273-327, 1996, Eds. Severian Damitriu, Marcel Dekker, NY. It is therefore a routine matter for one of ordinary skill in the art to isolate a population of peptides produced by a cell and determine the structure(s) of the glycans attached thereto. For example, efficient methods are available for (i) the splitting of glycosidic bonds either by chemical cleavage such as hydrolysis, acetolysis, hydrazinolysis, or by nitrous deamination; (ii) complete methylation followed by hydrolysis or methanolysis and by gas-liquid chromatography and mass spectroscopy of the partially methylated monosaccharides; and (iii) the definition of anomeric linkages between monosaccharides using exoglycosidases, which also provide insight into the primary glycan structure by sequential degradation. Fluorescent labeling and subsequent high performance liquid chromatography (HPLC), *e.g.*, normal phase HPLC (NP-HPLC), mass spectroscopy and nuclear magnetic resonance (NMR) spectrometry, *e.g.*, high field NMR, may also be used to determine glycan primary structure.

Kits and equipment for carbohydrate analysis are also commercially available. Fluorophore Assisted Carbohydrate Electrophoresis (FACE) is available from Glyko, Inc. (Novato, Calif.). In FACE analysis, glycoconjugates are released from the peptide with either Endo H or N-glycanase (PNGase F) for N-linked glycans, or hydrazine for Ser/Thr linked glycans. The glycan is then labeled at the reducing end with a fluorophore in a non-structure discriminating manner. The fluorophore labeled glycans are then separated in polyacrylamide gels based on the charge/mass ratio of the saccharide as well as the hydrodynamic volume. Images are taken of the gel under UV light and the composition of the glycans is determined by the migration distance as compared with the standards. Oligosaccharides can be sequenced in this manner by analyzing migration shifts due to the sequential removal of saccharides by exoglycosidase digestion.

All N-linked oligosaccharides have a common “pentasaccharide core” of $\text{Man}_3\text{GlcNAc}_2$. (“Man” refers to mannose; “Glc” refers to glucose; “NAc” refers to N-acetyl; and “GlcNAc” refers to N-acetylglucosamine). The pentasaccharide core is also referred to as the “trimannose core” or the “paucimannose core”.

N-glycans differ with respect to the presence of, and/or in the number of branches (also called “antennae”) comprising peripheral sugars such as N-acetylglucosamine, galactose, N-acetylgalactosamine, N-acetylneuraminic acid, fucose and sialic acid that are added to the $\text{Man}_3\text{GlcNAc}_2$ core structure. Optionally, this structure may also contain a core fucose molecule and/or a xylose molecule. For a review of standard glycobiology nomenclature see, *Essentials of Glycobiology* Varki *et al.* eds., 1999, CSHL Press, the contents of which are incorporated herein by reference.

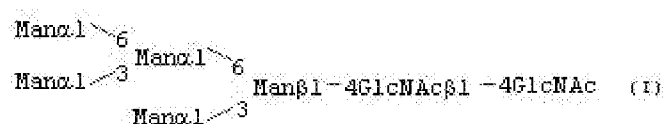
N-glycans are classified according to their branched constituents (*e.g.*, oligomannose-type, complex, or hybrid). An “oligomannose-type” or “high mannose-type” N-glycan has five or more mannose residues.

A “complex-type” N-glycan typically has at least one GlcNAc attached to the 1,3 mannose arm and at least one GlcNAc attached to the 1,6 mannose arm of a pentasaccharide core. Complex-type N-glycans may also have galactose (“Gal”) or N-acetylgalactosamine residues that are optionally modified with sialic acid or derivatives, *e.g.*, N-acetyl neuraminic acid. Complex-type N-glycans may also have intrachain substitutions comprising “bisecting” GlcNAc, and core fucose (“Fuc”). Complex N-glycans may also have multiple antennae on the pentasaccharide core and are, therefore, also referred to as “multiple antennary-type glycans.”

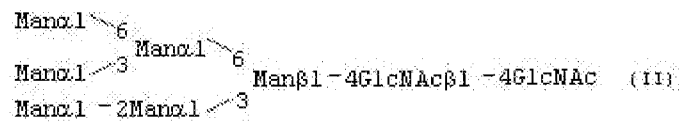
A “hybrid-type” N-glycan comprises at least one GlcNAc on the terminal of the 1,3 mannose arm of the pentasaccharide core and zero or more mannoses on the 1,6 mannose arm of the trimannose core.

The oligomannose-type structures that may be present within the compositions of the invention and/or may be used in the methods of the invention are referred to herein as “M5” or “Man 5 glycan”; “M6” or “Man 6 glycan”; “M7” or “Man 7 glycan”; “M8” or “Man 8 glycan”; and “M9” or “Man 9 glycan.”

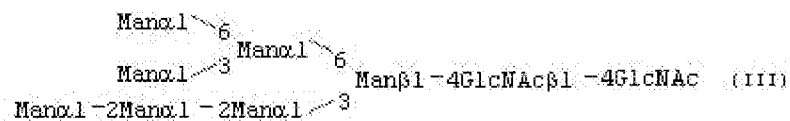
In one embodiment, an M5 oligomannose-type structure has the structure (I):



In one embodiment, an M6 oligomannose-type structure has the structure (II):

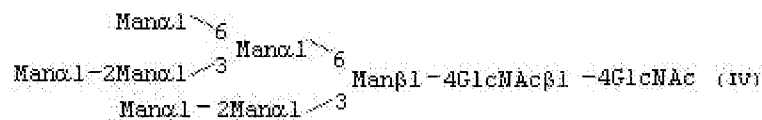


In one embodiment, an M7 oligomannose-type structure has the structure (III):

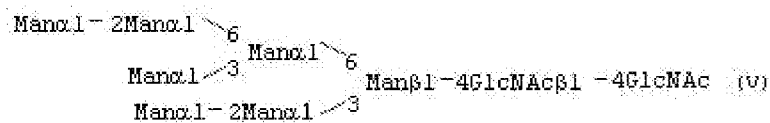


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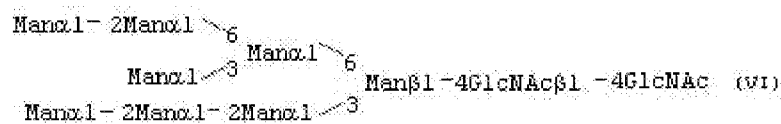
In another embodiment, an M7 oligomannose-type structure has the structure (IV):



In another embodiment, an M7 oligomannose-type structure has the structure (V):

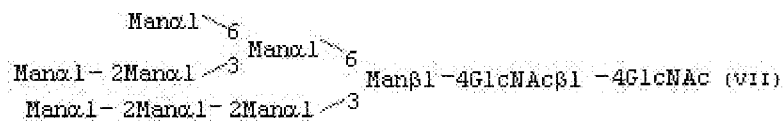


In one embodiment, an M8 oligomannose-type structure has the structure (VI):

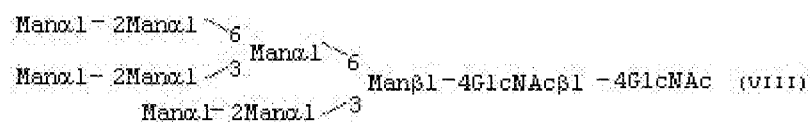


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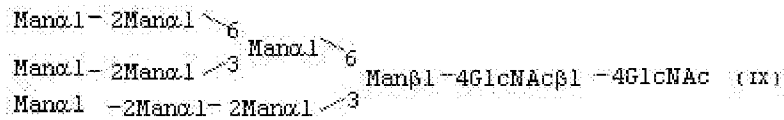
In another embodiment, an M8 oligomannose-type structure has the structure (VII):



In another embodiment, an M8 oligomannose-type structure has the structure (VIII):



In one embodiment, an M9 oligomannose-type structure has the structure (IX):



In one embodiment, the oligomannose-type structures that may be present within the compositions of the invention and/or may be used in the methods of the invention are

- 5 independently selected from the group consisting of Man 5 glycan, Man 6 glycan, Man 7 glycan, Man 8 glycan, and/or Man 9 glycan.

In one embodiment, a multiple antennary-type structure that may be present within the compositions of the invention and/or may be used in the methods of the invention is a “bianntennary oligosaccharide-type structure”. A “bianntennary oligosaccharide-type structure” is an N-linked glycan having two branches or arms, and a core fucose with zero, one or two galactose additions on the arms. In one embodiment, a “bianntennary oligosaccharide-type structure” that may be present within the compositions of the invention and/or may be used in the methods of the invention is bisected. In one embodiment, a “bianntennary oligosaccharide-type structure” that may be present within the compositions of the invention and/or may be used in the methods of the invention is a “fucosylated bianntennary oligosaccharide-type structure”, *e.g.*, comprises a core-substituted with fucose.

In one embodiment, a “fucosylated bianntennary oligosaccharide-type structure” that may be present within the compositions of the invention and/or may be used in the methods of the invention is an “asialo, fucosylated bianntennary oligosaccharide-type structure”, also referred to as an “asialo, bigalactosylated bianntennary, core-substituted with fucose”, referred to herein as “NA2F.”

In another embodiment, a “fucosylated bianntennary oligosaccharide-type structure” that may be present within the compositions of the invention and/or may be used in the methods of the invention is a asialo, agalacto, fucosylated bianntennary oligosaccharide-type structure, also referred to as an asialo, agalacto-, bianntennary, core-substituted with fucose, referred to herein as “NGA2F.”

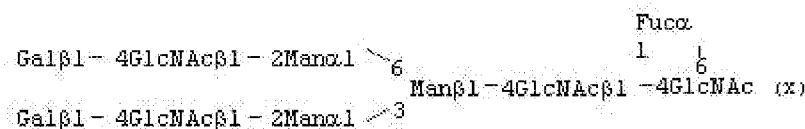
In another embodiment, a “fucosylated bianntennary oligosaccharide-type structure” that may be present within the compositions of the invention and/or may be used in the methods of the invention is a asialo, fucosylated bianntennary oligosaccharide-type structure,

also referred to as asialo, monogalactosylated biantennary, core-substituted with fucose, referred to herein as “NA1F.”

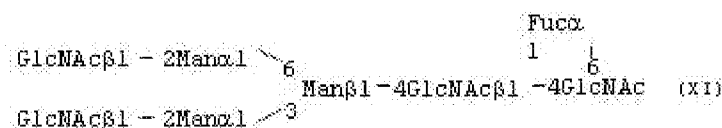
In another embodiment, a “fucosylated biantennary oligosaccharide-type structure” that may be present within the compositions of the invention and/or may be used in the methods of the invention is a asialo, agalacto, fucosylated biantennary, minus a bisecting N-acetylglucosamine oligosaccharide-type structure, also referred to as asialo, agalacto-, biantennary, core-substituted with fucose minus a bisecting N-acetylglucosamine, referred to herein as “NGA2F-GlcNAc.”

In yet another embodiment, a “fucosylated biantennary oligosaccharide-type structure” that may be present within the compositions of the invention and/or may be used in the methods of the invention is a asialo, monogalacto, fucosylated biantennary, minus a bisecting N-acetylglucosamine oligosaccharide-type structure, also referred to as asialo, monogalactosylated biantennary, core-substituted with fucose minus a bisecting N-acetylglucosamine, referred to herein as “NA1F-GlcNAc.”

In one embodiment, an NA2F fucosylated biantennary oligosaccharide-type structure has the structure (X):



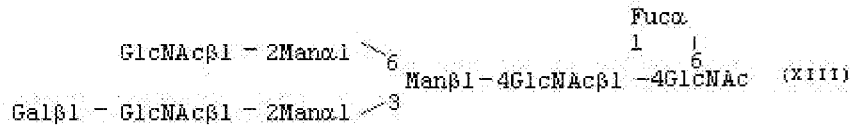
In one embodiment, an NGA2F fucosylated biantennary oligosaccharide-type structure has the structure (XI):



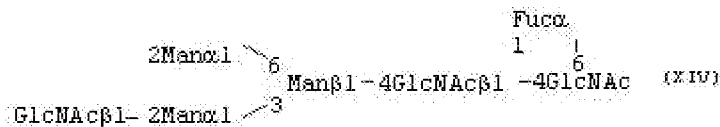
In one embodiment, an NA1F fucosylated biantennary oligosaccharide-type structure has the structure (XII):



oligosaccharide-type structure has the structure (XIV):



structure has the structure (XV):



As used herein, a “modulated glycosylation profile” includes a profile of a composition comprising a protein (*e.g.*, an antibody or DVD-Ig) which is modulated as compared to the glycosylation profile of a composition comprising that same protein produced by culturing a host cell expressing that protein in cell culture media which is not supplemented with a monosaccharide (*e.g.*, tagatose) and/or an oligosaccharide (*e.g.*, sucrose). The modulated glycosylation profile may include an overall increase in the level of mannosylated N-glycans and an overall decrease in the level of fucosylated N-glycans in the protein. For example, the overall level of mannosylated N-glycans in the protein may be increased by about 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99%. Ranges within one or more of the preceding values, *e.g.*, 1-10%, 1-15%,

1-20%, 1-25%, 1-30%, 1-35%, 1-40%, 1-41%, 1-42%, 1-43%, 1-44%, 1-45%, 1-46%, 1-47%, 1-48%, 1-49%, 1-50%, 2-10%, 2-15%, 2-20%, 2-25%, 2-30%, 2-35%, 2-40%, 2-41%, 2-42%, 2-43%, 2-44%, 2-45%, 2-46%, 2-47%, 2-48%, 2-49%, 2-50%, 3-10%, 3-15%, 3-20%, 3-25%, 3-30%, 3-35%, 3-40%, 3-41%, 3-42%, 3-43%, 3-44%, 3-45%, 3-46%, 3-47%, 3-48%, 3-49%, 3-50%, 4-10%, 4-15%, 4-20%, 4-25%, 4-30%, 4-35%, 4-40%, 4-41%, 4-42%, 4-43%, 4-44%, 4-45%, 4-46%, 4-47%, 4-48%, 4-49%, 4-50% or 1-99% are contemplated by the invention.

In another example, the overall level of mannosylated N-glycans comprises an increase in the amount or level of a high mannose N-glycan oligosaccharide. A high-mannose N-glycan has more than one mannose linked to the non-reducing terminal of the core structure. For example, the high mannose N-glycan oligosaccharide is selected from the group consisting of Man 5 glycan, Man 6 glycan, Man 7 glycan and Man 8 glycan. In one embodiment, the amount or level of at least one of Man 5 glycan, Man 6 glycan, Man 7 glycan and/or Man 8 glycan is increased by about 0.1%, 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99%. Ranges within one or more of the preceding values, *e.g.*, 0.1-5%, 0.1-10%, 0.1-15%, 0.1-20%, 0.1-21%, 0.1-22%, 0.1-23%, 0.1-24%, 0.1-25%, 0.1-26%, 0.1-27%, 0.1-28%, 0.1-29%, 0.1-30%, 0.1-35%, 0.1-40%, 0.1-41%, 0.1-42%, 0.1-43%, 0.1-44%, 0.1-45%, 0.1-46%, 0.1-47%, 0.1-48%, 0.1-49%, 0.1-50%, 1-5%, 1-10%, 1-15%, 1-20%, 1-21%, 1-22%, 1-23%, 1-24%, 1-25%, 1-26%, 1-27%, 1-28%, 1-29%, 1-30%, 1-35%, 1-40%, 1-41%, 1-42%, 1-43%, 1-44%, 1-45%, 1-46%, 1-47%, 1-48%, 1-49%, 1-50%, 2-5%, 2-10%, 2-15%, 2-20%, 2-21%, 2-22%, 2-23%, 2-24%, 2-25%, 2-26%, 2-27%, 2-28%, 2-29%, 2-30%, 2-35%, 2-40%, 2-41%, 2-42%, 2-43%, 2-44%, 2-45%, 2-46%, 2-47%, 2-48%, 2-49%, 2-50%, 3-5%, 3-10%, 3-15%, 3-20%, 3-21%, 3-22%, 3-24%, 3-25%, 3-26%, 3-27%, 3-28%, 2-29%, 3-30%, 3-35%, 3-40%, 3-41%, 3-42%, 3-43%, 3-44%, 3-45%, 3-46%, 3-47%, 3-48%, 3-49%, 3-50%, 4-5%, 4-10%, 4-15%, 4-20%, 4-25%, 4-30%, 4-35%, 4-40%, 4-41%, 4-42%, 4-43%, 4-44%, 4-45%, 4-46%, 4-47%, 4-48%, 4-49%, 4-50% or 0.1-99% are contemplated by the invention.

In another example, an overall decrease in the level of fucosylated N-glycans resulting from modulation of any one of the fucosylated glycan species such as NGA2F-GlcNAc, NGA2F, NA1F-GlcNAc, NA1F and/or NA2F is decreased by about 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99%. Ranges

within one or more of the preceding values, *e.g.*, 1-10%, 1-15%, 1-20%, 1-25%, 1-30%, 1-35%, 1-40%, 1-41%, 1-42%, 1-43%, 1-44%, 1-45%, 1-46%, 1-47%, 1-48%, 1-49%, 1-50%, 2-10%, 2-15%, 2-20%, 2-25%, 2-30%, 2-35%, 2-40%, 2-41%, 2-42%, 2-43%, 2-44%, 2-45%, 2-46%, 2-47%, 2-48%, 2-49%, 2-50%, 3-10%, 3-15%, 3-20%, 3-25%, 3-30%, 3-35%, 3-40%, 3-41%, 3-42%, 3-43%, 3-44%, 3-45%, 3-46%, 3-47%, 3-48%, 3-49%, 3-50%, 4-10%, 4-15%, 4-20%, 4-25%, 4-30%, 4-35%, 4-40%, 4-41%, 4-42%, 4-43%, 4-44%, 4-45%, 4-46%, 4-47%, 4-48%, 4-49%, 4-50%, 5-10%, 5-15%, 5-20%, 5-25%, 5-30%, 5-35%, 5-40%, 5-41%, 5-42%, 5-43, 5-44%, 5-45%, 5-46%, 5-47%, 5-48%, 5-49%, 5-50% or 1-99% are contemplated by the invention.

The term “level” with respect to protein such as an antibody, or antigen-binding fragment thereof, which is glycosylated at an N-linked glycosylation site on the Fc region in a composition refers to the relation of one glycoform in the composition to the whole of the glycoform levels in the composition and is expressed as a percentage of the whole, *e.g.*, 0-100%. The level in a composition may be an absolute amount as measured in molecules, moles, or weight percent.

Compositions comprising varying levels of glycoforms of a protein such as a human antibody, or antigen-binding fragment thereof, are useful in that by varying the glycoform compositions a desired characteristics, *e.g.*, rate of serum clearance or ADCC activity, may be achieved.

The methods of the invention can be used to produce compositions of any protein, such as a therapeutic protein, *e.g.*, an antibody, an antigen-binding portion thereof, a DVD-Ig, a TVD-Ig, a RAB or a half-body.

The term “antibody” includes an immunoglobulin molecule comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant region (CH). The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs,

arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

The term "antigen-binding portion" of an antibody (or "antibody portion") includes fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.*, in the case of Adalimumab, hTNF α). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment comprising the VL, VH, CL and CHI domains; (ii) a F(ab')₂ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment comprising the VH and CHI domains; (iv) a Fv fragment comprising the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) Nature 341:544-546, the entire teaching of which is incorporated herein by reference), which comprises a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VB regions pair to form monovalent molecules (known as single chain Fv (scFv); see, *e.g.*, Bird *et al.* (1988) Science 242:423-426; and Huston *et al.* (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883, the entire teachings of which are incorporated herein by reference). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. Other forms of single chain antibodies, such as diabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see, *e.g.*, Holliger, P., *et al.* (1993) Proc. Natl. Acad. Sci. USA 90:6444-6448; Poljak, R. J., *et al.* (1994) Structure 2:1121-1123, the entire teachings of which are incorporated herein by reference). Still further, an antibody or antigen-binding portion thereof may be part of a larger immunoadhesion molecule, formed by covalent or non-covalent association of the antibody or antibody portion with one or more other proteins or peptides. Examples of such immunoadhesion molecules include use of the streptavidin core region to make a tetrameric scFv molecule (Kipriyanov, S. M., *et al.* (1995) Human Antibodies and Hybridomas 6:93-101, the entire teaching of which is incorporated herein by

reference) and use of a cysteine residue, a marker peptide and a C-terminal polyhistidine tag to make bivalent and biotinylated scFv molecules (Kipriyanov, S. M., *et al.* (1994) *Mol Immunol.* 31:1047-1058, the entire teaching of which is incorporated herein by reference). Antibody portions, such as Fab and F(ab')₂ fragments, can be prepared from whole antibodies using conventional techniques, such as papain or pepsin digestion, respectively, of whole antibodies. Moreover, antibodies, antibody portions and immunoadhesion molecules can be obtained using standard recombinant DNA techniques, as described herein. In one aspect, the antigen binding fragments are complete domains or pairs of complete domains.

The term "human antibody" includes antibodies having variable and constant regions corresponding to human germline immunoglobulin sequences as described by Kabat *et al.* (See Kabat, *et al.* (1991) *Sequences of proteins of Immunological Interest*, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242). The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*), *e.g.*, in the CDRs and in particular CDR3. The mutations can be introduced using the "selective mutagenesis approach." The human antibody can have at least one position replaced with an amino acid residue, *e.g.*, an activity enhancing amino acid residue which is not encoded by the human germline immunoglobulin sequence. The human antibody can have up to twenty positions replaced with amino acid residues which are not part of the human germline immunoglobulin sequence. In other embodiments, up to ten, up to five, up to three or up to two positions are replaced. In one embodiment, these replacements are within the CDR regions. However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

The phrase "recombinant human antibody" includes human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies expressed using a recombinant expression vector transfected into a host cell, antibodies isolated from a recombinant, combinatorial human antibody library, antibodies isolated from an animal (*e.g.*, a mouse) that is transgenic for human immunoglobulin genes (see, *e.g.*, Taylor, L. D., *et al.* (1992) *Nucl. Acids Res.* 20:6287-6295, the entire teaching of which is incorporated herein by reference) or antibodies prepared, expressed, created or isolated by any other means that involves splicing of human immunoglobulin gene sequences to other DNA sequences. Such

recombinant human antibodies have variable and constant regions derived from human germline immunoglobulin sequences (see, Kabat, E. A., *et al.* (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242). In certain embodiments, however, such recombinant human antibodies are subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germline VH and VL sequences, may not naturally exist within the human antibody germline repertoire *in vivo*. In certain embodiments, however, such recombinant antibodies are the result of selective mutagenesis approach or back-mutation or both.

An "isolated antibody" includes an antibody that is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds hTNF α is substantially free of antibodies that specifically bind antigens other than hTNF α). An isolated antibody that specifically binds hTNF α may bind TNF α molecules from other species. Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals. A suitable anti-TNF α antibody is adalimumab.

As used herein, the term "adalimumab," also known by its trade name HUMIRA[®] (AbbVie) refers to a human IgG₁ antibody that binds human tumor necrosis factor α (TNF α). In general, the heavy chain constant domain 2 (CH2) of the adalimumab IgG-Fc region is glycosylated through covalent attachment of oligosaccharide at asparagine 297 (Asn-297). The light chain variable region of adalimumab is provided herein as SEQ ID NO:1, and the heavy chain variable region of adalimumab is provided herein as SEQ ID NO:2.

Adalimumab comprises a light chain variable region comprising a CDR1 of SEQ ID NO:7, a CDR2 of SEQ ID NO:5, and a CDR3 of SEQ ID NO:3. Adalimumab comprises a heavy chain variable region comprising a CDR1 of SEQ ID NO:8, a CDR2 of SEQ ID NO:6 and CDR3 of SEQ ID NO:4. The nucleic acid sequence of the light chain variable region is set forth in SEQ ID NO:9. The nucleic acid sequence of the heavy chain variable region is set forth in SEQ ID NO:10. The full length amino acid sequence of the light chain is set forth as SEQ ID NO:11 and the full length amino acid sequence of the heavy chain is set forth as SEQ ID NO:12. Adalimumab is described in U.S. Patent Nos. 6,090,382; 6,258,562; 6,509,015; 7,223,394; 7,541,031; 7,588,761; 7,863,426; 7,919,264; 8,197,813; 8,206,714; 8,216,583; 8,420,081; 8,092,998; 8,093,045; 8,187,836; 8,372,400; 8,034,906; 8,436,149; 8,231,876; 8,414,894; 8,372,401, the entire contents of each which are expressly incorporated herein by

reference in their entireties. Adalimumab is also described in the “Highlights of Prescribing Information” for HUMIRA® (adalimumab) Injection (Revised Jan. 2008) the contents of which are hereby incorporated herein by reference.

As used herein, a heavy chain antigen binding domain (referred to herein as VD or VDH) is intended to include a heavy chain variable domain, a dual heavy chain variable domain, a triple heavy chain variable domain, a light chain variable domain, a dual light chain variable domain, a triple light chain variable domain, a heavy chain variable domain in combination with a light chain variable domain, two heavy chain variable domains in combination with a light chain variable domain, a heavy chain variable domain in combination with two light chain variable domains, a domain antibody, a camelid antibody, a scFv, a receptor, and a scaffold antigen binding protein. It is understood that the heavy chain antigen binding domain may or may not bind an antigen independently of a paired light chain, dual light chain, or triple light chain, as appropriate, present on a second polypeptide of the binding proteins of the invention. For example, a domain antibody, a scFv, or a receptor would be expected to bind a target independent of any amino acid sequences on a second polypeptide claim. As the binding proteins of the invention form functional antigen binding sites, if the heavy chain antigen binding domain cannot specifically bind a target antigen independently (i.e., does not alone provide a functional antibody binding site), a second polypeptide should be present to provide a complementary light chain variable domain to provide a functional antibody binding site.

As used herein, a light chain antigen binding domain (referred to herein as VD or VDL) is intended to include a light chain variable domain, a dual light chain variable domain, a triple light chain variable domain, a heavy chain variable domain, a dual heavy chain variable domain, a triple heavy chain variable domain, a heavy chain variable domain in combination with a light chain variable domain, two heavy chain variable domains in combination with a light chain variable domain, a heavy chain variable domain in combination with two light chain variable domains, a camelid antibody, a domain antibody, a camelid antibody, a scFv, a receptor, and a scaffold antigen binding protein. It is understood that the light chain antigen binding domain may or may not bind an antigen independently of a paired heavy chain, dual heavy chain, or triple heavy chain, as appropriate, present on another polypeptide of the binding proteins of the invention. For example, a domain antibody, a scFv, or a receptor would be expected to bind a target independent of any amino acid sequences on a second polypeptide claim.

As used herein, "VD" alone can be understood to be either a heavy chain antigen binding domain or a light chain antigen binding domain unless otherwise clear from context.

As used herein, "Dual Variable Domain Immunoglobulin" or "DVD-IgTM" and the like are understood to include binding proteins having the structure schematically represented in Figure 19 and provided in US Patent Publications 20100260668 and 20090304693 both of which are incorporated herein by reference. DVDs may be monospecific, *i.e.*, bind one antigen, or multispecific, *i.e.* bind two or more antigens. A DVD-IgTM comprises a paired heavy chain DVD polypeptide and a light chain DVD polypeptide with each paired heavy and light chain providing two antigen binding sites. Each binding site includes a total of 6 CDRs involved in antigen binding per antigen binding site. A DVD-IgTM is typically has two arms bound to each other at least in part by dimerization of the CH3 domains, with each arm of the DVD being bispecific, providing an immunoglobulin with four binding sites.

A TVD-Ig is described in PCT Publication No. WO 2012/088290, the entire contents of which are incorporated herein by reference. A half-body is described in PCT Publication No. WO 2012/088302, the entire contents of which are incorporated herein by reference.

As used herein, the term "upstream process technology," in the context of protein, *e.g.*, antibody, preparation, refers to activities involving the production and collection of proteins (*e.g.* antibodies or DVD-Igs) from cells (*e.g.*, during cell culture of a protein with a modulated glycosylation profile). As used herein, the term "cell culture" refers to methods and techniques employed to generate and maintain a population of host cells capable of producing a recombinant protein with a modulated glycosylation profile, as well as the methods and techniques for optimizing the production and collection of the protein with a modulated glycosylation profile. For example, once an expression vector has been incorporated into an appropriate host, the host can be maintained under conditions suitable for high level expression of the relevant nucleotide coding sequences, and the collection and purification of the desired recombinant protein.

When using the cell culture techniques of the instant invention, the protein with a modulated glycosylation profile can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. In embodiments where the protein with a modulated glycosylation profile is produced intracellularly, the particulate debris, either host cells or lysed cells (*e.g.*, resulting from homogenization), can be removed by a variety of means, including but not limited to, by centrifugation or ultrafiltration. Where the protein with a

modulated glycosylation profile is secreted into the medium, supernatants from such expression systems can be first concentrated using a commercially available protein concentration filter, *e.g.*, an Amicon™ or Millipore Pellicon™ ultrafiltration unit

As used herein, the term “downstream process technology” refers to one or more techniques used after the upstream process technologies to purify the protein, *e.g.*, antibody, antigen-binding portion thereof, or DVD-Ig, of interest. For example, downstream process technology includes purification of the protein product, using, for example, affinity chromatography, including Protein A affinity chromatography, ion exchange chromatography, such as anion or cation exchange chromatography, hydrophobic interaction chromatography, displacement chromatography, multi-mode chromatography, continuous and recycle chromatography, viral filtration, depth filtration, ultrafiltration, diafiltration and centrifugation.

As used herein a “recombinant expression vector” can be any suitable recombinant expression vector, and can be used to transform or transfect any suitable host. For example, one of ordinary skill in the art would appreciate that transformation or transfection is a process by which exogenous nucleic acid such as DNA is introduced into a cell wherein the transformation or transfection process involves contacting the cell with the exogenous nucleic acid such as the recombinant expression vector as described herein. Non-limiting examples of such expression vectors are the pUC series of vectors (Fermentas Life Sciences), the pBluescript series of vectors (Stratagene, LaJolla, Calif.), the pET series of vectors (Novagen, Madison, Wis.), the pGEX series of vectors (Pharmacia Biotech, Uppsala, Sweden), and the pEX series vectors (Clontech, Palo Alto, Calif.).

The phrase “recombinant host cell” (or simply “host cell”) includes a cell into which a recombinant expression vector has been introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term “host cell” as used herein. In an embodiment, host cells include prokaryotic and eukaryotic cells selected from any of the Kingdoms of life. In another embodiment, eukaryotic cells include protist, fungal, plant and animal cells. In another embodiment, host cells include, but are not limited to, the prokaryotic cell line *E. coli*; mammalian cell lines CHO, HEK 293, COS, NS0, SP2 and PER.C6; the insect cell line Sf9; and the fungal cell *Saccharomyces cerevisiae*.

In certain embodiments, the host cells used in the methods of the present invention are prokaryote, yeast, or higher eukaryote cells. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, e.g., Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as Bacilli such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published Apr. 12, 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One suitable *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In certain embodiments, the host cells are eukaryotic microbes such as filamentous fungi or yeast. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such as, e.g., *K. lactis*, *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickerhamii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilae* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus*; *Yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida*; *Trichoderma reesei* (EP 244,234); *Neurospora crassa*; *Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, e.g., *Neurospora*, *Penicillium*, *Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger*.

In certain embodiments the host cells are derived from multicellular organisms. In particular embodiments, the cells are invertebrate cells from plant and insect cells. Non-limiting examples include cells derived from *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), *Bombyx mori*, cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized. As used herein, the term "recombinant protein" refers to a protein produced as the result of the transcription and translation of a gene carried on a recombinant expression vector that has been introduced into a host cell. In certain embodiments the recombinant protein is an antibody, preferably a chimeric, humanized, or fully human antibody. In certain embodiments the recombinant protein is an antibody of an isotype selected from group consisting of: IgG (e.g., IgG1, IgG2, IgG3, IgG4), IgM, IgA1, IgA2, IgD, or IgE. In certain embodiments the antibody molecule is a full-length antibody (e.g., an IgG1 or IgG4

immunoglobulin) or alternatively the antibody can be a fragment (e.g., an Fc fragment or a Fab fragment). In some embodiments, the recombinant protein is a DVD-Ig, a TVD-Ig, a RAB or a half-body.

In the methods of the invention, the host cells are cultured in media supplemented
5 with an oligosaccharide and/or a monosaccharide. As used herein, the term
“monosaccharide” refers to any of a class of carbohydrates that cannot be broken down to
simpler sugars by hydrolysis and that constitute the building blocks of oligosaccharides and
polysaccharides. Monosaccharides consist of at least three carbon atoms, one of which is
10 attached to an oxygen atom to form an aldehyde group (CHO) or a ketone, and the others of
which are each attached to a hydroxyl group (OH). A monosaccharide comprising three
carbons per molecule is referred to a triose. A monosaccharide comprising four carbons per
molecule is referred to as a tetrose. A monosaccharide comprising five carbons per molecule
is referred to as a pentose. A monosaccharide sugar containing six carbons per molecule is
15 referred to as a hexose. Monosaccharides can occur as chains or rings. Non-limiting examples
of monosaccharides include tagatose, glucose, galactose, ribose, fructose and xylose. In one
embodiment of the invention, the monosaccharide is not glucose.

As used herein the term “oligosaccharide” refers to a saccharide polymer containing a
small number of, generally two to ten, monosaccharides. The monosaccharide units are
bonded to each other by glycosidic linkages. Non-limiting examples of oligosaccharides
20 include sucrose, lactose, maltose, raffinose, trehalose, melibiose, maltotriose, gentianose and
maltopentalose.

The term “about”, as used herein, is intended to refer to ranges of approximately 0.1-
2.0% greater than or less than the referenced value. In certain circumstances, one of skill in
the art will recognize that, due to the nature of the referenced value, the term “about” can
25 mean more or less than a 0.1-2.0% deviation from that value.

The term “control”, as used herein, is intended to refer to a composition comprising a
protein produced by culturing a host cell expressing a protein in cell culture media which is
not supplemented with a monosaccharide and/or an oligosaccharide. For example, a control
may include a composition comprising a protein (e.g., an antibody) produced using the same
30 host cell line and the same recombinant expression vector under the same cell culture
conditions, including the same culture media, same culture vessel, same culture mode, same
culture temperature and same pH, but without monosaccharide or oligosaccharide

supplementation. For example, if antibody X is the antibody whose glycosylation profile is modulated using the methods of the invention, the control would be a composition comprising antibody X produced using the same host cell line and the same recombinant expression vector under the same cell culture conditions, including the same culture media, same culture vessel, same culture mode, same culture temperature and same pH, but without monosaccharide or oligosaccharide supplementation.

II. Modulation of Protein Glycosylation Using Monosaccharides and Oligosaccharides

Glycosylation

It is well known that the pattern of glycoforms that arise in recombinant proteins, including monoclonal antibodies, can be affected by culture conditions during production (Nam *et al.* (2008) *Biotechnol. Bioeng.* 100(6): 1178-92). Consistency in the quality of the glycoproteins is important as glycosylation may impact protein solubility, activity, and circulatory half-life. (Gawlitzek *et al.* (1995) *Biotechnol. Bioeng.* 46:536-544; and Hayter *et al.* (1992) *Biotechnol. Bioeng.* 39:327-335).

Post-translational modification of nascent recombinant proteins includes enzymatic glycosylation. The resulting proteins, bearing covalently linked oligosaccharide side chains, are known as glycosylated proteins or glycoproteins. Antibodies are glycoproteins with one or more carbohydrate residues in the Fc domain, as well as the variable domain. Carbohydrate residues in the Fc domain have an important effect on the effector function of the Fc domain, with minimal effect on antigen binding or half-life of the antibody (Jefferis, R. *Biotechnol. Prog.* (2005) 21:11-16). In contrast, glycosylation of the variable domain may have an effect on the antigen binding activity of the antibody. Glycosylation in the variable domain may also have a negative effect on antibody binding affinity, likely due to steric hindrance (Co, M.S. *et al.*, (1993) *Mol. Immunol.* 30:1361-1367), or result in increased affinity for the antigen (Wallick, S.C. *et al.*, (1988) *Exp. Med.* 168:1099-1109; Wright, A. *et al.*, (1991) *EMBO J.* 10:2717-2723).

Protein glycosylation depends on the amino acid sequence of the protein of interest, as well as the host cell in which the protein is expressed. Different organisms may produce different glycosylation enzymes (*e.g.*, glycosyltransferases and glycosidases), and have different substrates (nucleotide sugars) available. Due to such factors, protein glycosylation

pattern, and composition of glycosyl residues, may differ depending on the host system in which the particular protein is expressed. Glycosyl residues useful in the proteins produced using the methods of the present invention may include, but are not limited to, glucose, galactose, mannose, fucose, n-acetylglucosamine, NGA2F-GlcNAc, NGA2F, NA1F-

5 GlcNAc, NA1F, NA2F and sialic acid.

In one aspect of the present invention, the glycosylation of a protein, *e.g.*, antibody, antigen-binding portion thereof, or DVD-Ig, is modulated. Glycosylation can be modulated to, for example, increase the affinity of the antibody or antigen-binding portion for the antigen. Such carbohydrate modifications can be accomplished by, for example, altering
10 upstream process technologies, for example, recombinant host cell culture conditions by supplementing the cell culture media with an oligosaccharide (*e.g.*, sucrose) and/or a monosaccharide (*e.g.*, tagatose).

Additionally or alternatively, the present invention provides methods for modulating the glycosylation profile of a protein (*e.g.*, an antibody or DVD-Ig), such as modulating the
15 type of glycan species and/or the amount or level of glycan species present in the protein. For example, the methods of the present invention can be used to produce a hypofucosylated antibody having decreased amounts or levels of fucosyl residues. Such altered glycosylation patterns have been demonstrated to increase the ADCC ability of antibodies. In one embodiment, the amount of NGA2F species linked to the protein is decreased, for example,
20 the amount or level of NGA2F species linked to the protein is decreased by about 0.1%, 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 51%, 52%, 53%, 54%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99%. Ranges within one or more of the preceding values, *e.g.*, about 0.1% to 50%, 1% to 50%, 1% to 51%, 1% to 55%, 1% to 60%, 5% to 50%, 5% to
25 51%, 5% to 55%, 5% to 60%, 9% to 51%, 10% to 60%, or 0.1% to 99% are contemplated by the invention.

In another embodiment, the modulation of the glycosylation of the protein results in an increase in the amount or level of NGA2F-GlcNAc species linked to the protein, for example, the amount or level of NGA2F-GlcNAc species linked to the protein is increased by
30 about 0.1%, 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99%. Ranges within one or more of the preceding values,

e.g., about 0.1 % to 5%, 0.1% to 10%, 1% to 10%, 2% to 8%, 3% to 6%, 5% to 8% or 0.1% to 99% are contemplated by the invention.

In another embodiment, the modulation of the glycosylation of the protein results in an increase or a decrease in the amount or level of NA1F-GlcNAc species linked to the protein, for example, the amount or level of NA1F-GlcNAc species linked to the protein is increased or decreased by about 0.1%, 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99%. Ranges within one or more of the preceding values, *e.g.*, about 0.1-5%, 0.1-10% , 1% to 10%, 2% to 8%, 3% to 6%, 5% to 8% or 0.1% to 99% are contemplated by the invention.

In another embodiment, the modulation of the glycosylation of the protein results in an increase or a decrease in the amount or level of NA1F species linked to the protein, for example, the amount or level of NA1F species linked to the protein is increased or decreased by about 0.1%, 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99%. Ranges within one or more of the preceding values, *e.g.*, about 0.1-5%, 0.1-10% , 1% to 10%, 2% to 8%, 3% to 6%, 5% to 8% or 0.1% to 99% are contemplated by the invention.

In another embodiment, the modulation of the glycosylation of the protein results in an increase or a decrease in the amount or level of NA2F species linked to the protein, for example, the amount or level of NA2F species linked to the protein is increased or decreased by about 0.1%, 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99%. Ranges within one or more of the preceding values, *e.g.*, about 0.1-5%, 0.1-10% , 1% to 10%, 2% to 8%, 3% to 6%, 5% to 8% or 0.1% to 99% are contemplated by the invention.

In another embodiment, the modulation of the glycosylation of the protein results in an increase in the amount or level of NGA2F-GlcNAc, NA1F-GlcNAc, NA1F and/or NA2F species linked to the protein, for example the amount or level of NGA2F-GlcNAc, NA1F-GlcNAc, NA1F and/or NA2F linked to the protein is increased by about 0.1%, 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%

or 99%. Ranges within one or more of the preceding values, *e.g.*, about 0.1 % to 5%, 0.1% to 10%, 0.1% to 20%, 1% to 10%, 1% to 20%, 2% to 8%, 3% to 6%, 3% to 20%, 5% to 8%, 5% to 20% or 0.1% to 99%.

In another embodiment, the modulation of the glycosylation of the protein results in a decrease in the amount or level of NGA2F, NA1F-GlcNAc, NA1F and/or NA2F species linked to the protein, for example the amount or level of NGA2F, NA1F-GlcNAc, NA1F and/or NA2F linked to the protein is decreased by about 0.1%, 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%. Ranges within one or more of the preceding values, *e.g.*, about 0.1% to 5%, 0.1% to 10%, 0.1% to 20%, 0.1% to 30%, 0.1% to 40%, 0.1% to 50%, 0.1% to 60%, 0.1% to 65%, 1% to 10%, 1% to 20%, 1% to 30%, 1% to 40%, 1% to 50%, 1% to 60%, 1% to 65%, 5% to 10%, 5% to 20%, 5% to 30%, 5% to 40%, 5% to 50%, 5% to 60%, 5% to 65%, 10% to 20%, 10% to 30%, 10% to 40%, 10% to 50%, 10% to 60%, 10% to 65%, or 0.1 to 99%.

In another embodiment, the overall fucosylation level resulting from the modulation (*e.g.*, increase or decrease) of any one of the fucosylated glycan species such as NGA2F-GlcNAc, NGA2F, NA1F-GlcNAc, NA1F and/or NA2F is decreased by about 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99%. Ranges within one or more of the preceding values, *e.g.*, about 1-10%, 1-15%, 1-20%, 1-25%, 1-30%, 1-35%, 1-40%, 1-41%, 1-42%, 1-43%, 1-44%, 1-45%, 1-46%, 1-47%, 1-48%, 1-49%, 1-50%, 2-10%, 2-15%, 2-20%, 2-25%, 2-30%, 2-35%, 2-40%, 2-41%, 2-42%, 2-43%, 2-44%, 2-45%, 2-46%, 2-47%, 2-48%, 2-49%, 2-50%, 3-10%, 3-15%, 3-20%, 3-25%, 3-30%, 3-35%, 3-40%, 3-41%, 3-42%, 3-43%, 3-44%, 3-45%, 3-46%, 3-47%, 3-48%, 3-49%, 3-50%, 4-10%, 4-15%, 4-20%, 4-25%, 4-30%, 4-35%, 4-40%, 4-41%, 4-42%, 4-43%, 4-44%, 4-45%, 4-46%, 4-47%, 4-48%, 4-49%, 4-50%, 5-10%, 5-15%, 5-20%, 5-25%, 5-30%, 5-35%, 5-40%, 5-41%, 5-42%, 5-43, 5-44%, 5-45%, 5-46%, 5-47%, 5-48%, 5-49%, 5-50% or 1-99% are contemplated by the invention.

In another embodiment, the modulation of the glycosylation of the protein (*e.g.*, antibody or DVD-Ig) results in an increase in the amount or level of mannosylation, for example, the amount or level of mannosylation is increased by about 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99%. Ranges within one or

more of the preceding values, *e.g.*, about 1-5%, 1-10%, 1-15%, 1-20%, 1-25%, 1-30%, 1-35%, 1-40%, 1-41%, 1-42%, 1-43%, 1-44%, 1-45%, 1-46%, 1-47%, 1-48%, 1-49%, 1-50%, 2-5%, 2-10%, 2-15%, 2-20%, 2-25%, 2-30%, 2-35%, 2-40%, 2-41%, 2-42%, 2-43%, 2-44%, 2-45%, 2-46%, 2-47%, 2-48%, 2-49%, 2-50%, 3-5%, 3-10%, 3-15%, 3-20%, 3-25%, 3-30%, 3-35%, 3-40%, 3-41%, 3-42%, 3-43%, 3-44%, 3-45%, 3-46%, 3-47%, 3-48%, 3-49%, 3-50%, 4-5%, 4-10%, 4-15%, 4-20%, 4-25%, 4-30%, 4-35%, 4-40%, 4-41%, 4-42%, 4-43%, 4-44%, 4-45%, 4-46%, 4-47%, 4-48%, 4-49%, 4-50% or 1-99% are contemplated by the invention.

In another embodiment, the increase in mannosylation of the protein comprises an increase in the amount or level of a high mannose N-glycan oligosaccharide. A high-mannose N-glycan has more than one mannose linked to the non-reducing terminal of the core structure. For example, the high mannose N-glycan oligosaccharide is selected from the group consisting of Man 5 glycan, Man 6 glycan, Man 7 glycan and Man 8 glycan. In one embodiment, the amount or level of at least one of Man 5 glycan, Man 6 glycan, Man 7 glycan and/or Man 8 glycan is increased by about 0.1%, 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99%. Ranges within one or more of the preceding values, *e.g.*, about 0.1-5%, 0.1-10%, 0.1-15%, 0.1-20%, 0.1-21%, 0.1-22%, 0.1-23%, 0.1-24%, 0.1-25%, 0.1-26%, 0.1-27%, 0.1-28%, 0.1-29%, 0.1-30%, 0.1-35%, 0.1-40%, 0.1-41%, 0.1-42%, 0.1-43%, 0.1-44%, 0.1-45%, 0.1-46%, 0.1-47%, 0.1-48%, 0.1-49%, 0.1-50%, 1-5%, 1-10%, 1-15%, 1-20%, 1-21%, 1-22%, 1-23%, 1-24%, 1-25%, 1-26%, 1-27%, 1-28%, 1-29%, 1-30%, 1-35%, 1-40%, 1-41%, 1-42%, 1-43%, 1-44%, 1-45%, 1-46%, 1-47%, 1-48%, 1-49%, 1-50%, 2-5%, 2-10%, 2-15%, 2-20%, 2-21%, 2-22%, 2-23%, 2-24%, 2-25%, 2-26%, 2-27%, 2-28%, 2-29%, 2-30%, 2-35%, 2-40%, 2-41%, 2-42%, 2-43%, 2-44%, 2-45%, 2-46%, 2-47%, 2-48%, 2-49%, 2-50%, 3-5%, 3-10%, 3-15%, 3-20%, 3-21%, 3-22%, 3-24%, 3-25%, 3-26%, 3-27%, 3-28%, 2-29%, 3-30%, 3-35%, 3-40%, 3-41%, 3-42%, 3-43%, 3-44%, 3-45%, 3-46%, 3-47%, 3-48%, 3-49%, 3-50%, 4-5%, 4-10%, 4-15%, 4-20%, 4-25%, 4-30%, 4-35%, 4-40%, 4-41%, 4-42%, 4-43%, 4-44%, 4-45%, 4-46%, 4-47%, 4-48%, 4-49%, 4-50% or 0.1-99% are contemplated by the invention.

It is known to those skilled in the art that differing protein glycosylation profiles may result in differing protein characteristics. For instance, the efficacy of a therapeutic protein produced in a microorganism host, such as yeast, and glycosylated utilizing the yeast endogenous pathway may be reduced compared to that of the same protein expressed in a

mammalian cell, such as a CHO cell line. Such glycoproteins may also be immunogenic in humans and show reduced half-life *in vivo* after administration. Specific receptors in humans and other animals may recognize specific glycosyl residues and promote the rapid clearance of the protein from the bloodstream. Other adverse effects may include changes in protein
5 folding, solubility, susceptibility to proteases, trafficking, transport, compartmentalization, secretion, recognition by other proteins or factors, antigenicity, or allergenicity. Accordingly, using the methods of the invention, one of skill in the art may modulate the glycosylation profile of a protein, *e.g.*, an antibody or DVD-Ig to achieve a desired activity such as increased or decreased rate of clearance and/or increased ADCC activity.

10

Upstream Process Technologies

The methods of the present invention may be used to produce a protein (*e.g.*, an antibody, or antigen binding fragment thereof, or a DVD-Ig) with a modulated glycosylation profile. In one embodiment, the methods of the invention involve modification of the
15 conditions used during upstream protein production, such as recombinant cell culture conditions. For example, the methods of the invention comprise supplementing the recombinant cell culture media with a monosaccharide (*e.g.*, tagatose) and/or oligosaccharide (*e.g.*, sucrose) to modulate the glycosylation profile of the protein.

The upstream process technologies may be used alone or in combination with the
20 downstream process technologies described below.

In one embodiment, the methods described herein produce a protein with a modulated glycosylation profile wherein the overall fucosylation level resulting from the modulation of any one of the fucosylated glycan species such as NGA2F-GlcNAc, NGA2F, NA1F-GlcNAc, NA1F and/or NA2F, is decreased by about 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%,
25 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, and ranges within one or more of the preceding. In one aspect of this embodiment, the overall fucosylation level is decreased by about 1-10%, 1-15%, 1-20%, 1-25%, 1-30%, 1-35%, 1-40%, 1-41%, 1-42%, 1-43%, 1-44%,
1-45%, 1-46%, 1-47%, 1-48%, 1-49%, 1-50%, 2-10%, 2-15%, 2-20%, 2-25%, 2-30%, 2-
30 35%, 2-40%, 2-41%, 2-42%, 2-43%, 2-44%, 2-45%, 2-46%, 2-47%, 2-48%, 2-49%, 2-50%, 3-10%, 3-15%, 3-20%, 3-25%, 3-30%, 3-35%, 3-40%, 3-41%, 3-42%, 3-43%, 3-44%, 3-45%, 3-46%, 3-47%, 3-48%, 3-49%, 3-50% 4-10%, 4-15%, 4-20%, 4-25%, 4-30%, 4-35%,

4-40%, 4-41%, 4-42%, 4-43%, 4-44%, 4-45%, 4-46%, 4-47%, 4-48%, 4-49%, 4-50%, 5-10%, 5-15%, 5-20%, 5-25%, 5-30%, 5-35%, 5-40%, 5-41%, 5-42%, 5-43, 5-44%, 5-45%, 5-46%, 5-47%, 5-48%, 5-49%, 5-50% or 1-99% and ranges within one or more of the preceding.

5 In another embodiment, the methods described herein produce a protein with a modulated glycosylation profile wherein the overall mannosylation level resulting from the modulation of any one of the high mannose N-glycan oligosaccharides, such as Man 5 glycan, Man 6 glycan, Man 7 glycan or Man 8 glycan, is increased by about 0.1%, 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%,
 10 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, and ranges within one or more of the preceding. In one aspect of this embodiment, the overall high mannose N-glycan level is increased by about 0.1-5%, 0.1-10%, 0.1-15%, 0.1-20%, 0.1-21%, 0.1-22%, 0.1-23%, 0.1-24%, 0.1-25%, 0.1-26%, 0.1-27%, 0.1-28%, 0.1-29%, 0.1-30%, 0.1-35%, 0.1-40%, 0.1-41%, 0.1-42%, 0.1-43%, 0.1-44%, 0.1-45%, 0.1-46%, 0.1-47%, 0.1-48%,
 15 0.1-49%, 0.1-50%, 1-5%, 1-10%, 1-15%, 1-20%, 1-21%, 1-22%, 1-23%, 1-24%, 1-25%, 1-26%, 1-27%, 1-28%, 1-29%, 1-30%, 1-35%, 1-40%, 1-41%, 1-42%, 1-43%, 1-44%, 1-45%, 1-46%, 1-47%, 1-48%, 1-49%, 1-50%, 2-5%, 2-10%, 2-15%, 2-20%, 2-21%, 2-22%, 2-23%, 2-24%, 2-25%, 2-26%, 2-27%, 2-28%, 2-29%, 2-30%, 2-35%, 2-40%, 2-41%, 2-42%, 2-43%, 2-44%, 2-45%, 2-46%, 2-47%, 2-48%, 2-49%, 2-50%, 3-5%, 3-10%, 3-15%, 3-20%, 3-
 20 21%, 3-22%, 3-24%, 3-25%, 3-26%, 3-27%, 3-28%, 2-29%, 3-30%, 3-35%, 3-40%, 3-41%, 3-42%, 3-43%, 3-44%, 3-45%, 3-46%, 3-47%, 3-48%, 3-49%, 3-50%, 4-5%, 4-10%, 4-15%, 4-20%, 4-25%, 4-30%, 4-35%, 4-40%, 4-41%, 4-42%, 4-43%, 4-44%, 4-45%, 4-46%, 4-47%, 4-48%, 4-49%, 4-50% or 0.1-99%, and ranges within one or more of the preceding.

In certain embodiments, the methods described herein produce a protein, *e.g.*, an
 25 antibody, with a modulated glycosylation profile wherein the antibody's antibody-dependent cellular cytotoxicity (ADCC) response is increased by about 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, and ranges within one or more of the preceding. In one aspect of this embodiment, the antibody's ADCC response is increased by about 1-5%, 1-10%, 1-15%, 1-
 30 20%, 1-25%, 1-30%, 1-35%, 1-40%, 1-45%, 1-50%, 5-10%, 5-15%, 5-20%, 5-25%, 5-30%, 5-35%, 5-40%, 5-45%, 5-50%, 8-10%, 8-15%, 8-20%, 8-25%, 8-30%, 8-35%, 8-40%, 8-45%, 8-50%, 10-15%, 10-20%, 10-25%, 10-30%, 10-35%, 10-40%, 10-45%, 10-50%, 20-

25%, 20-30%, 20-35%, 20-40%, 20-45%, 20-50% or 1-99%, and ranges within one or more of the preceding.

As described herein, the host cell culture conditions can be modified as compared to conditions during production of the same protein without modulation of the glycosylation profile. In one embodiment, a protein with a modulated glycosylation profile is produced by culturing cells expressing the antibody, or antigen binding fragment thereof, or DVD-Ig in a cell culture media supplemented with an oligosaccharide (*e.g.*, sucrose) and/or a monosaccharide (*e.g.*, tagatose).

To express a protein with a modulated glycosylation profile (*e.g.*, an antibody, or antigen-binding fragment thereof, or DVD-Ig), DNAs encoding the protein, such as DNAs encoding partial or full-length light and heavy chains in the case of antibodies, are inserted into one or more expression vector such that the genes are operatively linked to transcriptional and translational control sequences. (See, *e.g.*, U.S. Pat. No. 6,090,382, the entire contents of which are incorporated herein by reference.) In this context, the term "operatively linked" is intended to mean that a gene encoding the protein is ligated into a vector such that transcriptional and translational control sequences within the vector serve their intended function of regulating the transcription and translation of the gene. The expression vector and expression control sequences are chosen to be compatible with the expression host cell used. In certain embodiments, the protein with a modulated glycosylation profile will comprising multiple polypeptides, such as the heavy and light chains of an antibody. Thus, in certain embodiments, genes encoding multiple polypeptides, such as antibody light chain genes and antibody heavy chain genes, can be inserted into a separate vector or, more typically, the genes are inserted into the same expression vector. Genes are inserted into expression vectors by standard methods (*e.g.*, ligation of complementary restriction sites on the gene fragment and vector, or blunt end ligation if no restriction sites are present). Prior to insertion of the gene or genes, the expression vector may already carry additional polypeptide sequences, such as, but not limited to, antibody constant region sequences. For example, one approach to converting the anti-TNF α antibody or anti-TNF α antibody-related VH and VL sequences to full-length antibody genes is to insert them into expression vectors already encoding heavy chain constant and light chain constant regions, respectively, such that the VH segment is operatively linked to the CH segment(s) within the vector and the VL segment is operatively linked to the CL segment within the vector. Additionally or alternatively, the recombinant expression vector can encode a signal peptide

that facilitates secretion of the protein from a host cell. The gene can be cloned into the vector such that the signal peptide is linked in-frame to the amino terminus of the gene. The signal peptide can be an immunoglobulin signal peptide or a heterologous signal peptide (*i.e.*, a signal peptide from a non-immunoglobulin protein).

5 In addition to protein coding genes, a recombinant expression vector can carry one or more regulatory sequence that controls the expression of the protein coding genes in a host cell. The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals) that control the transcription or translation of the protein coding genes. Such regulatory sequences are described, *e.g.*, in
10 Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990), the entire teaching of which is incorporated herein by reference. It will be appreciated by those skilled in the art that the design of the expression vector, including the selection of regulatory sequences may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. Suitable regulatory sequences
15 for mammalian host cell expression include viral elements that direct high levels of protein expression in mammalian cells, such as promoters and/or enhancers derived from cytomegalovirus (CMV) (such as the CMV promoter/enhancer), Simian Virus 40 (SV40) (such as the SV40 promoter/enhancer), adenovirus, (*e.g.*, the adenovirus major late promoter (AdMLP)) and polyoma. For further description of viral regulatory elements, and sequences
20 thereof, see, *e.g.*, U.S. Patent No. 5,168,062 by Stinski, U.S. Patent No. 4,510,245 by Bell *et al.* and U.S. Patent No. 4,968,615 by Schaffner *et al.*, the entire teachings of which are incorporated herein by reference.

A recombinant expression vector may also carry one or more additional sequences, such as a sequence that regulates replication of the vector in host cells (*e.g.*, origins of
25 replication) and/or a selectable marker gene. The selectable marker gene facilitates selection of host cells into which the vector has been introduced (see *e.g.*, U.S. Patents Nos. 4,399,216, 4,634,665 and 5,179,017, all by Axel *et al.*, the entire teachings of which are incorporated herein by reference). For example, typically the selectable marker gene confers resistance to drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has
30 been introduced. Suitable selectable marker genes include the dihydrofolate reductase (DHFR) gene (for use in dhfr- host cells with methotrexate selection/amplification) and the neo gene (for G418 selection).

An antibody, or antigen binding fragment thereof, to be used in the method of preparing a protein with a modulated glycosylation profile can be prepared by recombinant expression of immunoglobulin light and heavy chain genes in a host cell. To express an antibody recombinantly, a host cell is transfected with one or more recombinant expression vectors carrying DNA fragments encoding the immunoglobulin light and heavy chains of the antibody such that the light and heavy chains are expressed in the host cell and secreted into the medium in which the host cells are cultured, from which medium the antibodies can be recovered. Standard recombinant DNA methodologies are used to obtain antibody heavy and light chain genes, incorporate these genes into recombinant expression vectors and introduce the vectors into host cells, such as those described in Sambrook, Fritsch and Maniatis (eds), Molecular Cloning; A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989), Ausubel *et al.* (eds.) Current Protocols in Molecular Biology, Greene Publishing Associates, (1989) and in U.S. Patent Nos. 4,816,397 & 6,914,128, the entire teachings of which are incorporated herein.

For expression of a protein, for example, the light and heavy chains of an antibody, the expression vector(s) encoding the protein is (are) transfected into a host cell by standard techniques. The various forms of the term “transfection” are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, *e.g.*, electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. Although it is theoretically possible to express the proteins of the invention in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells, such as mammalian host cells, is suitable because such eukaryotic cells, and in particular mammalian cells, are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active protein. Prokaryotic expression of protein genes has been reported to be ineffective for production of high yields of active protein (Boss and Wood (1985) Immunology Today 6:12-13, the entire teaching of which is incorporated herein by reference).

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, *e.g.*, Enterobacteriaceae such as Escherichia, *e.g.*, E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, *e.g.*, Salmonella typhimurium, Serratia, *e.g.*, Serratia marcescans, and Shigella, as well as Bacilli such as B. subtilis and B. licheniformis (*e.g.*, B. licheniformis 41P

disclosed in DD 266,710 published Apr. 12, 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One suitable *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

5 In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for polypeptide encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such
10 as, *e.g.*, *K. lactis*, *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickerhamii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilae* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus*; *Yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida*; *Trichoderma reesei* (EP 244,234); *Neurospora crassa*; *Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, *e.g.*, *Neurospora*, *Penicillium*,
15 *Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger*.

Suitable host cells for the expression of proteins with modulated glycosylation profiles, for example, glycosylated antibodies, are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera*
20 *frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda*
25 cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

Mammalian cells can be used for expression and production of the protein compositions of the invention, however other eukaryotic cell types can also be employed in the context of the instant invention. See, *e.g.*, Winnacker, *From Genes to Clones*, VCH
30 Publishers, N.Y., N.Y. (1987). Suitable mammalian host cells for expressing recombinant proteins according to the invention include Chinese Hamster Ovary (CHO cells) (including dhfr- CHO cells, described in Urlaub and Chasin, (1980) PNAS USA 77:4216-4220, used with a DHFR selectable marker, *e.g.*, as described in Kaufman and Sharp (1982) Mol. Biol.

159:601-621, the entire teachings of which are incorporated herein by reference), NS0 myeloma cells, COS cells and SP2 cells. When recombinant expression vectors encoding protein genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or secretion of the antibody into the culture medium in which the host cells are grown. Other examples of useful mammalian host cell lines are 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.*, J. Gen Virol. 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub *et al.*, Proc. Natl. Acad. Sci. USA 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod. 23:243-251 (1980)); 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 liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, Annals N.Y. Acad. Sci. 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2), the entire teachings of which are incorporated herein by reference.

Host cells are transformed with the above-described expression or cloning vectors for protein production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

The host cells used to produce a protein may be cultured in a variety of media which are supplemented in accordance with the present invention. Commercially available media such as Ham's F10™ (Sigma), Minimal Essential Medium™ (MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium™ (DMEM), (Sigma), Iscove's Modified Dulbecco's Medium, Minimal Essential Medium-alpha. (MEM-alpha), DME/F12, alpha MEM, Basal Medium Eagle with Earle's BSS, DMEM high Glucose, with L-Glutamine, DMEM high glucose, without L-Glutamine, DMEM low Glucose, without L-Glutamine, DMEM:F12 1:1, with L-Glutamine, GMEM (Glasgow's MEM), GMEM with L-glutamine, Grace's Complete Insect Medium, Grace's Insect Medium, without FBS, Ham's F-10, with L-Glutamine, Ham's F-12, with L-Glutamine, IMDM with HEPES and L-Glutamine, IMDM with HEPES and without L-Glutamine, IPL-41 Insect Medium, L-15 (Leibovitz)(2.times.),

without L-Glutamine or Phenol Red, L- 15 (Leibovitz), without L-Glutamine, McCoy's 5A Modified Medium, Medium 199, MEM Eagle, without L-Glutamine or Phenol Red (2.times.), MEM Eagle-Earle's BSS, with L-glutamine, MEM Eagle-Earle's BSS, without L-Glutamine, MEM Eagle-Hanks BSS, without L-Glutamine, NCTC-109, with L-Glutamine, 5 Richter's CM Medium, with L- Glutamine, RPMI 1640 with HEPES, L-Glutamine and/or Penicillin-Streptomycin, RPMI 1640, with L-Glutamine, RPMI 1640, without L-Glutamine, Schneider's Insect Medium are suitable for culturing host cells. In addition, any of the media described in Ham *et al.*, Meth. Enz. 58:44 (1979), Barnes *et al.*, Anal. Biochem. 102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 10 90/03430; WO 87/00195; or U.S. Pat. No. Re. 30,985 may be used as culture media for the host cells, the entire teachings of which are incorporated herein by reference.

Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such 15 as adenosine and thymidine), antibiotics (such as gentamycin drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the 20 host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

Host cells can also be used to produce portions of intact proteins, for example, antibodies, including Fab fragments or scFv molecules. It is understood that variations on the above procedure are within the scope of the present invention. For example, in certain embodiments it may be desirable to transfect a host cell with DNA encoding either the light 25 chain or the heavy chain (but not both) of an antibody. Recombinant DNA technology may also be used to remove some or all of the DNA encoding either or both of the light and heavy chains that is not necessary for binding to an antigen. The molecules expressed from such truncated DNA molecules are also encompassed by the antibodies of the invention. In addition, bifunctional antibodies may be produced in which one heavy and one light chain are 30 an antibody of the invention and the other heavy and light chain are specific for an antigen other than the target antibody, depending on the specificity of the antibody of the invention, by crosslinking an antibody of the invention to a second antibody by standard chemical crosslinking methods.

In a suitable system for recombinant expression of a protein, for example, an antibody, or antigen-binding portion thereof, or a DVD-Ig, a recombinant expression vector encoding the protein, for example, both an antibody heavy chain and an antibody light chain, is introduced into dhfr-CHO cells by calcium phosphate-mediated transfection. Within the
5 recombinant expression vector, the protein gene(s) are each operatively linked to CMV enhancer/AdMLP promoter regulatory elements to drive high levels of transcription of the gene(s). The recombinant expression vector also carries a DHFR gene, which allows for selection of CHO cells that have been transfected with the vector using methotrexate selection/amplification. The selected transformant host cells are cultured to allow for
10 expression of the protein, for example, the antibody heavy and light chains, and intact protein, for example, an antibody, is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture the host cells and recover the protein from the culture medium.

15 When using recombinant techniques, the protein, for example, antibodies or antigen binding fragments thereof, can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. In one aspect, if the protein is produced intracellularly, as a first step, the particulate debris, either host cells or lysed cells (*e.g.*, resulting from homogenization), can be removed, *e.g.*, by centrifugation or ultrafiltration. Where the protein
20 is secreted into the medium, supernatants from such expression systems can be first concentrated using a commercially available protein concentration filter, *e.g.*, an Amicon™ or Millipore Pellicon™ ultrafiltration unit.

Some antibodies can be secreted directly from the cell into the surrounding growth media; others are made intracellularly. For antibodies made intracellularly, the first step of a
25 purification process typically involves: lysis of the cell, which can be done by a variety of methods, including mechanical shear, osmotic shock, or enzymatic treatments. Such disruption releases the entire contents of the cell into the homogenate, and in addition produces subcellular fragments that are difficult to remove due to their small size. These are generally removed by differential centrifugation or by filtration. Where the antibody is
30 secreted, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, *e.g.*, an Amicon™ or Millipore Pellicon™ ultrafiltration unit. Where the antibody is secreted into the medium, the recombinant host cells can also be separated from the cell culture medium, *e.g.*, by tangential

flow filtration. Antibodies can be further recovered from the culture medium using the antibody purification methods of the invention.

In accordance with the present invention, modulation of the glycosylation profile of the protein (*e.g.*, antibody or DVD-Ig) produced by recombinant cell culture can be achieved by supplementation of the cell culture media with sucrose and/or tagatose. Specific host cell culture conditions can be used with various cultivation methods including, but not limited to, batch, fed-batch, chemostat and perfusion, and with various cell culture equipment including, but not limited to, shake flasks with or without suitable agitation, spinner flasks, stirred bioreactors, airlift bioreactors, membrane bioreactors, reactors with cells retained on a solid support or immobilized/entrapped as in microporous beads, and any other configuration appropriate for optimal growth and productivity of the desired host cell line.

Supplementation with Monosaccharides and/or Oligosaccharides to Modulate the Glycosylation Profile of the Expressed Protein

The present invention relates to modulation of the glycosylation profile in mammalian cell culture processes using cell culture component such as monosaccharide and/or oligosaccharide supplementation. These nutrients are also important for ensuring both robust cell growth and production of glycoproteins. In the present invention these components are utilized to affect the profile of glycosylation of the glycoprotein. For example, but not by way of limitation, by adjusting the concentration of one or both of these sugars the glycosylation profile can be modulated. Thus, the present invention provides methods to modulate the glycosylation profile introduced by upstream process technologies to achieve desired product glycosylation profiles.

In certain embodiments, a protein with a modulated glycosylation profile is prepared by supplementation of cell culture media with monosaccharides (*e.g.*, tagatose) and/or oligosaccharides (*e.g.*, sucrose). For example, supplementation with sucrose and/or tagatose results in a significant increase in non-fully processed N-glycans, including high mannose N-glycan species. This is consistent with the abrogation of the N-glycan biosynthetic pathway at the enzymatic reaction steps shown in Figure 2, which results in the accumulation of these particular N-glycans. For some particular recombinant glycoproteins, a high mannose isoform is a desired product quality attribute (Walsh, G. *et al.*, (2006) Nat. Biotechnol. 24(10):1241-52). In addition, as a result of the increase in mannosylation, the levels of

fucosylation are significantly decreased. The addition of fucose to N-glycans has been shown to reduce antibody dependent cellular cytotoxicity (ADCC) (Kanda Y. *et al.*, (2007)

Glycobiology, 17(1):104-18; Shields R.L., *et al.*, (2002) J. Biol. Chem. 2002. **277**(30):

26733-26740). Thus, where the ADCC response is the principle therapeutic mechanism of

5 antibody activity, the provision of methods for the preparation of recombinant protein therapeutics with a glycosylation profile characterized by decreased fucosylation, are beneficial.

In certain embodiments, the cell culture media is supplemented with one or more monosaccharides or oligosaccharides in order to modulate the glycosylation profile of the
10 protein (*e.g.*, an antibody, of antigen binding fragment thereof, or a DVD-Ig). In one embodiment the monosaccharide is tagatose. In one embodiment the cell culture media is supplemented with about 1 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, 10 mM, 15 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, 90 mM or 100 mM tagatose. In particular embodiments, the cell culture media is supplemented with about 1 mM, 10 mM, 30
15 mM, 50 mM or 70 mM tagatose.

In another embodiment the oligosaccharide is sucrose. In one embodiment the cell culture media is supplemented with about 1 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, 10 mM, 15 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, 90 mM or 100 mM sucrose. In particular embodiments, the cell culture media is supplemented with about 1 mM,
20 7 mM, 10 mM, 15 mM, 30 mM, 50 mM or 70 mM sucrose.

In certain embodiments, the cell culture media is supplemented with one or more monosaccharides or oligosaccharides in an amount effective to modulate the glycosylation profile of the protein such that the overall fucosylation amount or level, resulting from the modulation of at least one of the fucosylated glycan species such as NGA2F-GlcNAc,
25 NGA2F, NA1F-GlcNAc, NA1F and/or NA2F, is decreased by about 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, and ranges within one or more of the preceding. In one aspect of this embodiment, the overall fucosylation amount or level is decreased by about 1-10%, 1-15%, 1-20%, 1-25%, 1-30%, 1-35%, 1-40%, 1-41%,
30 1-42%, 1-43%, 1-44%, 1-45%, 1-46%, 1-47%, 1-48%, 1-49%, 1-50%, 2-10%, 2-15%, 2-20%, 2-25%, 2-30%, 2-35%, 2-40%, 2-41%, 2-42%, 2-43%, 2-44%, 2-45%, 2-46%, 2-47%, 2-48%, 2-49%, 2-50%, 3-10%, 3-15%, 3-20%, 3-25%, 3-30%, 3-35%, 3-40%, 3-41%, 3-42%, 3-43%, 3-44%, 3-45%, 3-46%, 3-47%, 3-48%, 3-49%, 3-50%, 4-10%, 4-15%, 4-20%,

4-25%, 4-30%, 4-35%, 4-40%, 4-41%, 4-42%, 4-43%, 4-44%, 4-45%, 4-46%, 4-47%, 4-48%, 4-49%, 4-50%, 5-10%, 5-15%, 5-20%, 5-25%, 5-30%, 5-35%, 5-40%, 5-41%, 5-42%, 5-43%, 5-44%, 5-45%, 5-46%, 5-47%, 5-48%, 5-49%, 5-50% or 1-99%, and ranges within one or more of the preceding.

5 In certain embodiments, the cell culture media is supplemented with one or more monosaccharides or oligosaccharides in an amount effective to modulate the glycosylation profile of the protein such that the overall mannosylation amount or level, resulting from the modulation of at least one of the high mannose N-glycan oligosaccharides, such as Man 5 glycan, Man 6 glycan, Man 7 glycan or Man 8 glycan, is increased by about 0.1%, 1%, 1.2%,
 10 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, and ranges within one or more of the preceding. In one aspect of this embodiment, the overall high mannose N-glycan amount or level is increased by about 0.1-5%, 0.1-10%, 0.1-15%, 0.1-20%, 0.1-21%, 0.1-22%, 0.1-23%, 0.1-24%, 0.1-25%, 0.1-26%, 0.1-27%, 0.1-28%, 0.1-29%,
 15 0.1-30%, 0.1-35%, 0.1-40%, 0.1-41%, 0.1-42%, 0.1-43%, 0.1-44%, 0.1-45%, 0.1-46%, 0.1-47%, 0.1-48%, 0.1-49%, 0.1-50%, 1-5%, 1-10%, 1-15%, 1-20%, 1-21%, 1-22%, 1-23%, 1-24%, 1-25%, 1-26%, 1-27%, 1-28%, 1-29%, 1-30%, 1-35%, 1-40%, 1-41%, 1-42%, 1-43%, 1-44%, 1-45%, 1-46%, 1-47%, 1-48%, 1-49%, 1-50%, 2-5%, 2-10%, 2-15%, 2-20%, 2-21%, 2-22%, 2-23%, 2-24%, 2-25%, 2-26%, 2-27%, 2-28%, 2-29%, 2-30%, 2-35%, 2-40%, 2-41%, 2-42%, 2-43%, 2-44%, 2-45%, 2-46%, 2-47%, 2-48%, 2-49%, 2-50%, 3-5%, 3-10%, 3-15%, 3-20%, 3-21%, 3-22%, 3-24%, 3-25%, 3-26%, 3-27%, 3-28%, 2-29%, 3-30%, 3-35%, 3-40%, 3-41%, 3-42%, 3-43%, 3-44%, 3-45%, 3-46%, 3-47%, 3-48%, 3-49%, 3-50%, 4-5%, 4-10%, 4-15%, 4-20%, 4-25%, 4-30%, 4-35%, 4-40%, 4-41%, 4-42%, 4-43%, 4-44%, 4-45%, 4-46%, 4-47%, 4-48%, 4-49%, 4-50% or 0.1-99%, and ranges within one or more of
 25 the preceding.

In certain embodiments, the cell culture media is supplemented with one or more monosaccharides or oligosaccharides in an amount effective to modulate the glycosylation profile of the protein, *e.g.*, an antibody, such that the antibody's antibody-dependent cellular cytotoxicity (ADCC) response is increased by about 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%,
 30 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, and ranges within one or more of the preceding. In one aspect of this embodiment, the antibody's ADCC response is increased by about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, or by about 1-5%, 1-10%, 1-15%, 1-20%, 1-25%, 1-30%, 1-35%, 1-40%, 1-

45%, 1-50%, 5-10%, 5-15%, 5-20%, 5-25%, 5-30%, 5-35%, 5-40%, 5-45%, 5-50%, 8-10%, 8-15%, 8-20%, 8-25%, 8-30%, 8-35%, 8-40%, 8-45%, 8-50%, 10-15%, 10-20%, 10-25%, 10-30%, 10-35%, 10-40%, 10-45%, 10-50%, 20-25%, 20-30%, 20-35%, 20-40%, 20-45%, 20-50% or 1-99%, and ranges within one or more of the preceding.

5 In certain embodiments, the cell culture media is supplemented, for example, at the start of culture, or in a fed-batch or in a continuous manner. The feed amounts may be calculated to achieve a certain concentration based on off-line or on-line measurements. The addition of one or more supplements may be based on measured glycosylation profiles. The resulting media can be used in various cultivation methods including, but not limited to,
10 batch, fed-batch, chemostat and perfusion, and with various cell culture equipment including, but not limited to, shake flasks with or without suitable agitation, spinner flasks, stirred bioreactors, airlift bioreactors, membrane bioreactors, reactors with cells retained on a solid support or immobilized/entrapped as in microporous beads, incubation vessels, microtiter plates, capillaries, multi-well plates and any other configuration appropriate for optimal
15 growth and productivity of the desired host cell line. Additional cell culture equipment may be used such as fermentor tanks, air lifts, culture flasks, spinner flasks, microcarriers, fluidized beds, hollow fibers, roller bottles or packed beds. In addition, the harvest criterion for these cultures may be chosen, for example, based on choice of harvest viability or culture duration, to further optimize a certain targeted glycosylation profiles.

20

Down Stream Process Technologies

The protein compositions of the invention may be purified using downstream process technologies (*e.g.*, purification or concentration), following production using the upstream process technologies of the present invention. For example, once a clarified solution or
25 mixture comprising the protein with a modulated glycosylation profile, *e.g.*, an antibody or DVD-Ig, has been obtained, separation of the protein from process-related impurities, such as the other proteins produced by the host cell, as well as product-related substances, such as acidic or basic variants, is performed. In certain embodiments, the initial steps of the purification methods involve the clarification and primary recovery of an antibody or DVD-Ig from a
30 sample matrix by methods such as centrifugation, depth filtration and/or viral inactivation/reduction. In certain non-limiting embodiments, further separation is performed using cation exchange chromatography, anion exchange chromatography, and/or multi-mode

chromatography. In certain embodiments, a combination of one or more different purification techniques, including affinity separation step(s), ion exchange separation step(s), mixed-mode step(s), and/or hydrophobic interaction separation step(s) can also be employed. Such additional purification steps separate mixtures of proteins on the basis of their charge, degree of hydrophobicity, and/or size. Continuous and recycle chromatography are also applicable to chromatography methods where the protein with a modulated glycosylation profile is collected in the unbound fraction during chromatography or where the protein is first bound to the chromatography resin and subsequently recovered by washing the media with conditions that elute the bound component. Numerous chromatography resins are commercially available for each of these techniques, allowing accurate tailoring of the purification scheme to the particular protein involved. Each of the separation methods allow proteins to either traverse at different rates through a column, achieving a physical separation that increases as they pass further through the column, or to adhere selectively to a separation resin (or medium). The proteins are then differentially eluted using different eluents. In some cases, the protein with a modulated glycosylation profile is separated from impurities when the impurities specifically adhere to the column's resin and the protein does not, *i.e.*, the protein is contained in the effluent, while in other cases the protein will adhere to the column's resin, while the impurities and/or product-related substances are extruded from the column's resin during a wash cycle. Following chromatographic polishing steps the protein compositions of the invention may be further purified using viral filtration. Ultrafiltration and/or diafiltration may be used to further concentrate and formulate the protein, *e.g.*, an antibody or DVD-Ig product.

The glycosylation profile of the protein prepared by the methods of the invention can be analyzed using methods well known to those skilled in the art, *e.g.*, removal and derivatization of N-glycans followed by NP-HPLC analysis, weak cation exchange chromatography (WCX), capillary isoelectric focusing (cIEF), size-exclusion chromatography, Poros A HPLC Assay, Host cell Protein ELISA, DNA assay, and western blot analysis.

III. Methods of Treatment Using Proteins with Modulated Glycosylation Profiles of the Invention

The compositions comprising a protein with a modulated glycosylation profile, for example a protein such as an antibody, antigen-binding portion thereof, or a DVD-Ig, with a decreased fucosylation level or amount and/or an increased mannosylation level or amount, of the invention may be used to treat any disorder in a subject for which the therapeutic protein (*e.g.*, an antibody, or an antigen binding fragment thereof, or a DVD-Ig) comprised in the composition is appropriate for treating.

A "disorder" is any condition that would benefit from treatment with the therapeutic protein with a modulated glycosylation profile. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the subject to the disorder in question. In the case of an anti-TNF α antibody, or antigen binding fragment thereof, such as adalimumab, a therapeutically effective amount of the composition comprising a protein with a modulated glycosylation profile may be administered to treat a disorder in which TNF α activity is detrimental.

A disorder in which TNF α activity is detrimental includes a disorder in which inhibition of TNF α activity is expected to alleviate the symptoms and/or progression of the disorder. Such disorders may be evidenced, for example, by an increase in the concentration of TNF α in a biological fluid of a subject suffering from the disorder (*e.g.*, an increase in the concentration of TNF α in serum, plasma, synovial fluid, *etc.* of the subject), which can be detected, for example, using an anti-TNF α antibody.

TNF α has been implicated in the pathophysiology of a wide variety of a TNF α -related disorders including sepsis, infections, autoimmune diseases, transplant rejection and graft-versus-host disease (see *e.g.*, Moeller, A., *et al.* (1990) *Cytokine* 2:162-169; U.S. Patent No. 5,231,024 to Moeller *et al.*; European Patent Publication No. 260 610 B1 by Moeller, A., *et al.* Vasilli, P. (1992) *Annu. Rev. Immunol.* 10:411-452; Tracey, K.J. and Cerami, A. (1994) *Annu. Rev. Med.* 45:491-503). Accordingly, the protein with a modulated glycosylation profile of the invention may be used to treat an autoimmune disease, such as rheumatoid arthritis, juvenile idiopathic arthritis, or psoriatic arthritis, an intestinal disorder, such as Crohn's disease or ulcerative colitis, a spondyloarthropathy, such as ankylosing spondylitis, or a skin disorder, such as psoriasis.

Disorders in which TNF α activity is detrimental are well known in the art and described in detail in U.S. Patent No. 8,231,876 and U.S. Patent No. 6,090,382, the entire contents of each of which are expressly incorporated herein by reference. In one embodiment, "a disorder in which TNF α activity is detrimental" includes sepsis (including

5 septic shock, endotoxic shock, gram negative sepsis and toxic shock syndrome), autoimmune diseases (including rheumatoid arthritis, rheumatoid spondylitis, osteoarthritis and gouty arthritis, allergy, multiple sclerosis, autoimmune diabetes, autoimmune uveitis, nephrotic syndrome, multisystem autoimmune diseases, lupus (including systemic lupus, lupus nephritis and lupus cerebritis), Crohn's disease and autoimmune hearing loss), infectious

10 diseases (including malaria, meningitis, acquired immune deficiency syndrome (AIDS), influenza and cachexia secondary to infection), allograft rejection and graft versus host disease, malignancy, pulmonary disorders (including adult respiratory distress syndrome (ARDS), shock lung, chronic pulmonary inflammatory disease, pulmonary sarcoidosis, pulmonary fibrosis, silicosis, idiopathic interstitial lung disease and chronic obstructive

15 airway disorders (COPD), such as asthma), intestinal disorders (including inflammatory bowel disorders, idiopathic inflammatory bowel disease, Crohn's disease and Crohn's disease-related disorders (including fistulas in the bladder, vagina, and skin; bowel obstructions; abscesses; nutritional deficiencies; complications from corticosteroid use; inflammation of the joints; erythem nodosum; pyoderma gangrenosum; lesions of the eye,

20 Crohn's related arthralgias, fistulizing Crohn's indeterminant colitis and pouchitis), cardiac disorders (including ischemia of the heart, heart insufficiency, restenosis, congestive heart failure, coronary artery disease, angina pectoris, myocardial infarction, cardiovascular tissue damage caused by cardiac arrest, cardiovascular tissue damage caused by cardiac bypass, cardiogenic shock, and hypertension, atherosclerosis, cardiomyopathy, coronary artery

25 spasm, coronary artery disease, valvular disease, arrhythmias, and cardiomyopathies), spondyloarthropathies (including ankylosing spondylitis, psoriatic arthritis/spondylitis, enteropathic arthritis, reactive arthritis or Reiter's syndrome, and undifferentiated spondyloarthropathies), metabolic disorders (including obesity and diabetes, including type 1 diabetes mellitus, type 2 diabetes mellitus, diabetic neuropathy, peripheral neuropathy,

30 diabetic retinopathy, diabetic ulcerations, retinopathy ulcerations and diabetic macrovasculopathy), anemia, pain (including acute and chronic pains, such as neuropathic pain and post-operative pain, chronic lower back pain, cluster headaches, herpes neuralgia, phantom limb pain, central pain, dental pain, opioid-resistant pain, visceral pain, surgical pain, bone injury pain, pain during labor and delivery, pain resulting from burns, including

sunburn, post partum pain, migraine, angina pain, and genitourinary tract-related pain including cystitis), hepatic disorders (including hepatitis, alcoholic hepatitis, viral hepatitis, alcoholic cirrhosis, α_1 antitrypsin deficiency, autoimmune cirrhosis, cryptogenic cirrhosis, fulminant hepatitis, hepatitis B and C, and steatohepatitis, cystic fibrosis, primary biliary cirrhosis, sclerosing cholangitis and biliary obstruction), skin and nail disorders (including psoriasis (including chronic plaque psoriasis, guttate psoriasis, inverse psoriasis, pustular psoriasis and other psoriasis disorders), pemphigus vulgaris, scleroderma, atopic dermatitis (eczema), sarcoidosis, erythema nodosum, hidradenitis suppurative, lichen planus, Sweet's syndrome, scleroderma and vitiligo), vasculitides (including Behcet's disease), and other disorders, such as juvenile rheumatoid arthritis (JRA), endometriosis, prostatitis, choroidal neovascularization, sciatica, Sjogren's syndrome, uveitis, wet macular degeneration, osteoporosis, osteoarthritis, active axial spondyloarthritis and non-radiographic axial spondyloarthritis.

As used herein, the term "subject" is intended to include living organisms, *e.g.*, prokaryotes and eukaryotes. Examples of subjects include mammals, *e.g.*, humans, dogs, cows, horses, pigs, sheep, goats, cats, mice, rabbits, rats, and transgenic non-human animals. In specific embodiments of the invention, the subject is a human.

As used herein, the term "treatment" or "treat" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder, as well as those in which the disorder is to be prevented.

In one embodiment, the invention provides a method of administering a composition comprising a protein with a modulated glycosylation profile, such as an anti-TNF α antibody, or antigen binding fragment thereof, to a subject such that TNF α activity is inhibited or a disorder in which TNF α activity is detrimental is treated. In one embodiment, the TNF α is human TNF α and the subject is a human subject. In one embodiment, the anti-TNF α antibody is adalimumab, also referred to as HUMIRA[®].

The compositions comprising a protein with a modulated glycosylation profile can be administered by a variety of methods known in the art. Exemplary routes/modes of administration include subcutaneous injection, intravenous injection or infusion. In certain aspects, a composition comprising a protein with a modulated glycosylation profile may be orally administered. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results.

Dosage regimens may be adjusted to provide the optimum desired response (*e.g.*, a therapeutic or prophylactic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. In certain
5 embodiments it is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit comprising a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical
10 carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic or prophylactic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

An exemplary, non-limiting range for a therapeutically or prophylactically effective
15 amount of a composition comprising a protein with a modulated glycosylation profile of the invention is 0.01-20 mg/kg, or 1-10 mg/kg, or 0.3-1 mg/kg. With respect to a composition comprising a protein such as an anti-TNF α antibody with a modulated glycosylation profile, or antigen-binding portion thereof, such as adalimumab, an exemplary dose is 40 mg every other week. In some embodiments, in particular for treatment of ulcerative colitis or Crohn's
20 disease, an exemplary dose includes an initial dose (Day 1) of 160 mg (*e.g.*, four 40 mg injections in one day or two 40 mg injections per day for two consecutive days), a second dose two weeks later of 80 mg, and a maintenance dose of 40 mg every other week beginning two weeks later. Alternatively, for psoriasis for example, a dosage can include an 80 mg initial dose followed by 40 mg every other week starting one week after the initial dose.

25 It is to be noted that dosage values may vary with the type and severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended
30 to limit the scope or practice of the claimed composition.

IV. Pharmaceutical Formulations Containing Compositions Comprising Proteins with Modulated Glycosylation Profiles of the Invention

The present invention further provides preparations and formulations comprising compositions comprising a protein with a modulated glycosylation profile, for example a protein such as an antibody, antigen-binding portion thereof, or a DVD-Ig, with a decreased fucosylation level or amount and/or an increased mannosylation level or amount. It should be understood that any of the compositions comprising the proteins with modulated glycosylation profiles, such as antibodies, antibody fragments and DVD-Igs described herein, may be formulated or prepared as described below. In one embodiment, the antibody is an anti-TNF α antibody, or antigen-binding portion thereof.

In certain embodiments, the compositions comprising a protein with a modulated glycosylation profile, of the invention may be formulated with a pharmaceutically acceptable carrier as pharmaceutical (therapeutic) compositions, and may be administered by a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. The term “pharmaceutically acceptable carrier” means one or more non-toxic materials that do not interfere with the effectiveness of the biological activity of the active ingredients. Such preparations may routinely contain salts, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. Such pharmaceutically acceptable preparations may also routinely contain compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration into a human. The term “carrier” denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being co-mingled with the protein with a modulated glycosylation profile (*e.g.*, antibodies or DVD-Igs) of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

The compositions comprising a protein with a modulated glycosylation profile, of the invention are present in a form known in the art and acceptable for therapeutic uses. In one embodiment, a formulation of the compositions comprising a protein with a modulated glycosylation profile, of the invention is a liquid formulation. In another embodiment, a formulation of the compositions comprising a protein with a modulated glycosylation profile, of the invention is a lyophilized formulation. In a further embodiment, a formulation of the compositions comprising a protein with a modulated glycosylation profile, of the invention is

a reconstituted liquid formulation. In one embodiment, a formulation of the compositions comprising a protein with a modulated glycosylation profile, of the invention is a stable liquid formulation. In one embodiment, a liquid formulation of the compositions comprising a protein with a modulated glycosylation profile, of the invention is an aqueous formulation.

5 In another embodiment, the liquid formulation is non-aqueous. In a specific embodiment, a liquid formulation of the compositions comprising a protein with a modulated glycosylation profile, of the invention is an aqueous formulation wherein the aqueous carrier is distilled water.

The formulations of the compositions comprising a protein with a modulated glycosylation profile (*e.g.*, an antibody or a DVD-Ig) in a concentration resulting in a w/v appropriate for a desired dose. The protein with a modulated glycosylation profile may be present in the formulation at a concentration of about 1 mg/ml to about 500 mg/ml, *e.g.*, at a concentration of at least 1 mg/ml, at least 5 mg/ml, at least 10 mg/ml, at least 15 mg/ml, at least 20 mg/ml, at least 25 mg/ml, at least 30 mg/ml, at least 35 mg/ml, at least 40 mg/ml, at least 45 mg/ml, at least 50 mg/ml, at least 55 mg/ml, at least 60 mg/ml, at least 65 mg/ml, at least 70 mg/ml, at least 75 mg/ml, at least 80 mg/ml, at least 85 mg/ml, at least 90 mg/ml, at least 95 mg/ml, at least 100 mg/ml, at least 105 mg/ml, at least 110 mg/ml, at least 115 mg/ml, at least 120 mg/ml, at least 125 mg/ml, at least 130 mg/ml, at least 135 mg/ml, at least 140 mg/ml, at least 150 mg/ml, at least 200 mg/ml, at least 250 mg/ml, or at least 300 mg/ml.

20 In a specific embodiment, a formulation of compositions comprising a protein with a modulated glycosylation profile, of the invention comprises at least about 100 mg/ml, at least about 125 mg/ml, at least 130 mg/ml, or at least about 150 mg/ml of protein with a modulated glycosylation profile (*e.g.*, an antibody or DVD-Ig) of the invention.

In one embodiment, the concentration of a protein with a modulated glycosylation profile (*e.g.*, antibody or DVD-Ig), which is included in the formulation of the invention, is between about 1 mg/ml and about 25 mg/ml, between about 1 mg/ml and about 200 mg/ml, between about 25 mg/ml and about 200 mg/ml, between about 50 mg/ml and about 200 mg/ml, between about 75 mg/ml and about 200 mg/ml, between about 100 mg/ml and about 200 mg/ml, between about 125 mg/ml and about 200 mg/ml, between about 150 mg/ml and about 200 mg/ml, between about 25 mg/ml and about 150 mg/ml, between about 50 mg/ml and about 150 mg/ml, between about 75 mg/ml and about 150 mg/ml, between about 100 mg/ml and about 150 mg/ml, between about 125 mg/ml and about 150 mg/ml, between about 25 mg/ml and about 125 mg/ml, between about 50 mg/ml and about 125 mg/ml, between about 75 mg/ml and about 125 mg/ml, between about 100 mg/ml and about 125 mg/ml,

between about 25 mg/ml and about 100 mg/ml, between about 50 mg/ml and about 100 mg/ml, between about 75 mg/ml and about 100 mg/ml, between about 25 mg/ml and about 75 mg/ml, between about 50 mg/ml and about 75 mg/ml, or between about 25 mg/ml and about 50 mg/ml.

5 In a specific embodiment, a formulation of the compositions comprising a protein with a modulated glycosylation profile of the invention comprises between about 90 mg/ml and about 110 mg/ml or between about 100 mg/ml and about 210 mg/ml of a protein with a modulated glycosylation profile (*e.g.*, an antibody or DVD-Ig).

10 The formulations of the compositions comprising a protein with a modulated glycosylation profile of the invention comprising a protein (*e.g.*, an antibody or DVD-Ig) may further comprise one or more active compounds as necessary for the particular indication being treated, typically those with complementary activities that do not adversely affect each other. Such additional active compounds are suitably present in combination in amounts that are effective for the purpose intended.

15 The formulations of the compositions comprising a protein with a modulated glycosylation profile may be prepared for storage by mixing the protein (*e.g.*, antibody or DVD-Ig) having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers, including, but not limited to buffering agents, saccharides, salts, surfactants, solubilizers, polyols, diluents, binders, stabilizers, salts, lipophilic solvents,
20 amino acids, chelators, preservatives, or the like (Goodman and Gilman's *The Pharmacological Basis of Therapeutics*, 12th edition, L. Brunton, *et al.* and *Remington's Pharmaceutical Sciences*, 16th edition, Osol, A. Ed. (1999)), in the form of lyophilized formulations or aqueous solutions at a desired final concentration. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations
25 employed, and include buffers such as histidine, phosphate, citrate, glycine, acetate and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low
30 molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including trehalose, glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol;

salt-forming counter-ions such as sodium; metal complexes (*e.g.*, Zn-protein complexes); and/or non-ionic surfactants such as TWEEN, polysorbate 80, PLURONICS™ or polyethylene glycol (PEG).

The buffering agent may be histidine, citrate, phosphate, glycine, or acetate. The
5 saccharide excipient may be trehalose, sucrose, mannitol, maltose or raffinose. The surfactant may be polysorbate 20, polysorbate 40, polysorbate 80, or Pluronic F68. The salt may be NaCl, KCl, MgCl₂, or CaCl₂

The formulations of the compositions comprising a protein with a modulated glycosylation profile of the invention may include a buffering or pH adjusting agent to
10 provide improved pH control. A formulation of the invention may have a pH of between about 3.0 and about 9.0, between about 4.0 and about 8.0, between about 5.0 and about 8.0, between about 5.0 and about 7.0, between about 5.0 and about 6.5, between about 5.5 and about 8.0, between about 5.5 and about 7.0, or between about 5.5 and about 6.5. In a further embodiment, a formulation of the invention has a pH of about 3.0, about 3.5, about 4.0, about
15 4.5, about 5.0, about 5.1, about 5.2, about 5.3, about 5.4, about 5.5, about 5.6, about 5.7, about 5.8, about 5.9, about 6.0, about 6.1, about 6.2, about 6.3, about 6.4, about 6.5, about 6.6, about 6.7, about 6.8, about 6.9, about 7.0, about 7.5, about 8.0, about 8.5, or about 9.0. In a specific embodiment, a formulation of the invention has a pH of about 6.0. One of skill in the art understands that the pH of a formulation generally should not be equal to the
20 isoelectric point of the particular a protein (*e.g.*, antibody or DVD-Ig) to be used in the formulation.

Typically, the buffering agent is a salt prepared from an organic or inorganic acid or base. Representative buffering agents include, but are not limited to, organic acid salts such as salts of citric acid, ascorbic acid, gluconic acid, carbonic acid, tartaric acid, succinic acid,
25 acetic acid, or phthalic acid; Tris, tromethamine hydrochloride, or phosphate buffers. In addition, amino acid components can also function in a buffering capacity. Representative amino acid components which may be utilized in the formulations of the invention as buffering agents include, but are not limited to, glycine and histidine. In certain embodiments, the buffering agent is chosen from histidine, citrate, phosphate, glycine, and
30 acetate. In a specific embodiment, the buffering agent is histidine. In another specific embodiment, the buffering agent is citrate. In yet another specific embodiment, the buffering agent is glycine. The purity of the buffering agent should be at least 98%, or at least 99%, or at least 99.5%. As used herein, the term “purity” in the context of histidine and glycine refers

to chemical purity of histidine or glycine as understood in the art, *e.g.*, as described in The Merck Index, 13th ed., O'Neil *et al.* ed. (Merck & Co., 2001).

Buffering agents are typically used at concentrations between about 1 mM and about 200 mM or any range or value therein, depending on the desired ionic strength and the

5 buffering capacity required. The usual concentrations of conventional buffering agents employed in parenteral formulations can be found in: Pharmaceutical Dosage Form: Parenteral Medications, Volume 1, 2nd Edition, Chapter 5, p. 194, De Luca and Boylan, "Formulation of Small Volume Parenterals", Table 5: Commonly used additives in Parenteral Products. In one embodiment, the buffering agent is at a concentration of about 1 mM, or of
10 about 5 mM, or of about 10 mM, or of about 15 mM, or of about 20 mM, or of about 25 mM, or of about 30 mM, or of about 35 mM, or of about 40 mM, or of about 45 mM, or of about 50 mM, or of about 60 mM, or of about 70 mM, or of about 80 mM, or of about 90 mM, or of about 100 mM. In one embodiment, the buffering agent is at a concentration of 1 mM, or of 5 mM, or of 10 mM, or of 15 mM, or of 20 mM, or of 25 mM, or of 30 mM, or of 35 mM, or
15 of 40 mM, or of 45 mM, or of 50 mM, or of 60 mM, or of 70 mM, or of 80 mM, or of 90 mM, or of 100 mM. In a specific embodiment, the buffering agent is at a concentration of between about 5 mM and about 50 mM. In another specific embodiment, the buffering agent is at a concentration of between 5 mM and 20 mM.

In certain embodiments, the formulation of the compositions comprising a protein
20 with a modulated glycosylation profile of the invention comprises histidine as a buffering agent. In one embodiment the histidine is present in the formulation of the invention at a concentration of at least about 1 mM, at least about 5 mM, at least about 10 mM, at least about 20 mM, at least about 30 mM, at least about 40 mM, at least about 50 mM, at least about 75 mM, at least about 100 mM, at least about 150 mM, or at least about 200 mM
25 histidine. In another embodiment, a formulation of the invention comprises between about 1 mM and about 200 mM, between about 1 mM and about 150 mM, between about 1 mM and about 100 mM, between about 1 mM and about 75 mM, between about 10 mM and about 200 mM, between about 10 mM and about 150 mM, between about 10 mM and about 100 mM, between about 10 mM and about 75 mM, between about 10 mM and about 50 mM, between
30 about 10 mM and about 40 mM, between about 10 mM and about 30 mM, between about 20 mM and about 75 mM, between about 20 mM and about 50 mM, between about 20 mM and about 40 mM, or between about 20 mM and about 30 mM histidine. In a further embodiment, the formulation comprises about 1 mM, about 5 mM, about 10 mM, about 20 mM, about 25 mM, about 30 mM, about 35 mM, about 40 mM, about 45 mM, about 50 mM,

about 60 mM, about 70 mM, about 80 mM, about 90 mM, about 100 mM, about 150 mM, or about 200 mM histidine. In a specific embodiment, a formulation may comprise about 10 mM, about 25 mM, or no histidine.

The formulations of the compositions comprising a protein with a modulated glycosylation profile of the invention may comprise a carbohydrate excipient. Carbohydrate excipients can act, *e.g.*, as viscosity enhancing agents, stabilizers, bulking agents, solubilizing agents, and/or the like. Carbohydrate excipients are generally present at between about 1% to about 99% by weight or volume, *e.g.*, between about 0.1% to about 20%, between about 0.1% to about 15%, between about 0.1% to about 5%, , between about 1% to about 20%, between about 5% to about 15%, between about 8% to about 10%, between about 10% and about 15%, between about 15% and about 20%, between 0.1% to 20%, between 5% to 15%, between 8% to 10%, between 10% and 15%, between 15% and 20%, between about 0.1% to about 5%, between about 5% to about 10%, or between about 15% to about 20%. In still other specific embodiments, the carbohydrate excipient is present at 1%, or at 1.5%, or at 2%, or at 2.5%, or at 3%, or at 4%, or at 5%, or at 10%, or at 15%, or at 20%.

Carbohydrate excipients suitable for use in the formulations of the invention include, but are not limited to, monosaccharides such as fructose, maltose, galactose, glucose, D-mannose, sorbose, and the like; disaccharides, such as lactose, sucrose, trehalose, cellobiose, and the like; polysaccharides, such as raffinose, melezitose, maltodextrins, dextrans, starches, and the like; and alditols, such as mannitol, xylitol, maltitol, lactitol, xylitol sorbitol (glucitol) and the like. In one embodiment, the carbohydrate excipients for use in the present invention are chosen from, sucrose, trehalose, lactose, mannitol, and raffinose. In a specific embodiment, the carbohydrate excipient is trehalose. In another specific embodiment, the carbohydrate excipient is mannitol. In yet another specific embodiment, the carbohydrate excipient is sucrose. In still another specific embodiment, the carbohydrate excipient is raffinose. The purity of the carbohydrate excipient should be at least 98%, or at least 99%, or at least 99.5%.

In a specific embodiment, the formulations of the compositions comprising a protein with a modulated glycosylation profile of the invention may comprise trehalose. In one embodiment, a formulation of the invention comprises at least about 1%, at least about 2%, at least about 4%, at least about 8%, at least about 20%, at least about 30%, or at least about 40% trehalose. In another embodiment, a formulation of the invention comprises between about 1% and about 40%, between about 1% and about 30%, between about 1% and about 20%, between about 2% and about 40%, between about 2% and about 30%, between about

2% and about 20%, between about 4% and about 40%, between about 4% and about 30%, or between about 4% and about 20% trehalose. In a further embodiment, a formulation of the invention comprises about 1%, about 2%, about 4%, about 6%, about 8%, about 15%, about 20%, about 30%, or about 40% trehalose. In a specific embodiment, a formulation of the invention comprises about 4%, about 6% or about 15% trehalose.

In certain embodiments, a formulation of the compositions comprising a protein with a modulated glycosylation profile of the invention comprises an excipient. In a specific embodiment, a formulation of the invention comprises at least one excipient chosen from: sugar, salt, surfactant, amino acid, polyol, chelating agent, emulsifier and preservative. In one embodiment, a formulation of the invention comprises a salt, *e.g.*, a salt selected from: NaCl, KCl, CaCl₂, and MgCl₂. In a specific embodiment, the formulation comprises NaCl.

A formulation of the compositions comprising a protein with a modulated glycosylation profile of the invention may comprise at least about 10 mM, at least about 25 mM, at least about 50 mM, at least about 75 mM, at least about 80 mM, at least about 100 mM, at least about 125 mM, at least about 150 mM, at least about 175 mM, at least about 200 mM, or at least about 300 mM sodium chloride (NaCl). In a further embodiment, the formulation may comprise between about 10 mM and about 300 mM, between about 10 mM and about 200 mM, between about 10 mM and about 175 mM, between about 10 mM and about 150 mM, between about 25 mM and about 300 mM, between about 25 mM and about 200 mM, between about 25 mM and about 175 mM, between about 25 mM and about 150 mM, between about 50 mM and about 300 mM, between about 50 mM and about 200 mM, between about 50 mM and about 175 mM, between about 50 mM and about 150 mM, between about 75 mM and about 300 mM, between about 75 mM and about 200 mM, between about 75 mM and about 175 mM, between about 75 mM and about 150 mM, between about 100 mM and about 300 mM, between about 100 mM and about 200 mM, between about 100 mM and about 175 mM, or between about 100 mM and about 150 mM sodium chloride. In a further embodiment, the formulation may comprise about 10 mM, about 25 mM, about 50 mM, about 75 mM, about 80 mM, about 100 mM, about 125 mM, about 150 mM, about 175 mM, about 200 mM, or about 300 mM sodium chloride.

A formulation of the compositions comprising a protein with a modulated glycosylation profile of the invention may also comprise an amino acid, *e.g.*, lysine, arginine, glycine, histidine or an amino acid salt. The formulation may comprise at least about 1 mM, at least about 10 mM, at least about 25 mM, at least about 50 mM, at least about 100 mM, at least about 150 mM, at least about 200 mM, at least about 250 mM, at least about 300 mM, at

least about 350 mM, or at least about 400 mM of an amino acid. In another embodiment, the formulation may comprise between about 1 mM and about 100 mM, between about 10 mM and about 150 mM, between about 25 mM and about 250 mM, between about 25 mM and about 300 mM, between about 25 mM and about 350 mM, between about 25 mM and about 400 mM, between about 50 mM and about 250 mM, between about 50 mM and about 300 mM, between about 50 mM and about 350 mM, between about 50 mM and about 400 mM, between about 100 mM and about 250 mM, between about 100 mM and about 300 mM, between about 100 mM and about 400 mM, between about 150 mM and about 250 mM, between about 150 mM and about 300 mM, or between about 150 mM and about 400 mM of an amino acid. In a further embodiment, a formulation of the invention comprises about 1 mM, 1.6 mM, 25 mM, about 50 mM, about 100 mM, about 150 mM, about 200 mM, about 250 mM, about 300 mM, about 350 mM, or about 400 mM of an amino acid.

The formulations of the compositions comprising a protein with a modulated glycosylation profile of the invention may further comprise a surfactant. The term “surfactant” as used herein refers to organic substances having amphipathic structures; namely, they are composed of groups of opposing solubility tendencies, typically an oil-soluble hydrocarbon chain and a water-soluble ionic group. Surfactants can be classified, depending on the charge of the surface-active moiety, into anionic, cationic, and nonionic surfactants. Surfactants are often used as wetting, emulsifying, solubilizing, and dispersing agents for various pharmaceutical compositions and preparations of biological materials. Pharmaceutically acceptable surfactants like polysorbates (*e.g.*, polysorbates 20 or 80); polyoxamers (*e.g.*, poloxamer 188); Triton; sodium octyl glycoside; lauryl-, myristyl-, linoleyl-, or stearyl-sulfobetaine; lauryl-, myristyl-, linoleyl- or stearyl-sarcosine; linoleyl-, myristyl-, or cetyl-betaine; lauroamidopropyl-, cocamidopropyl-, linoleamidopropyl-, myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-betaine (*e.g.*, lauroamidopropyl); myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-dimethylamine; sodium methyl cocoyl-, or disodium methyl oleyl-aurate; and the MONAQUA™ series (Mona Industries, Inc., Paterson, N.J.), polyethyl glycol, polypropyl glycol, and copolymers of ethylene and propylene glycol (*e.g.*, PLURONICS™, PF68, etc.), can optionally be added to the formulations of the invention to reduce aggregation. In one embodiment, a formulation of the invention comprises Polysorbate 20, Polysorbate 40, Polysorbate 60, or Polysorbate 80. Surfactants are particularly useful if a pump or plastic container is used to administer the formulation. The presence of a pharmaceutically acceptable surfactant mitigates the propensity for the protein to aggregate. The formulations

may comprise a polysorbate which is at a concentration ranging from between about 0.001% to about 1%, or about 0.001% to about 0.1%, or about 0.01% to about 0.1%. In other specific embodiments, the formulations of the invention comprise a polysorbate which is at a concentration of 0.001%, or 0.002%, or 0.003%, or 0.004%, or 0.005%, or 0.006%, or 0.007%, or 0.008%, or 0.009%, or 0.01%, or 0.015%, or 0.02%.

The formulations of the compositions comprising a protein with a modulated glycosylation profile of the invention may optionally further comprise other common excipients and/or additives including, but not limited to, diluents, binders, stabilizers, lipophilic solvents, preservatives, adjuvants, or the like. Pharmaceutically acceptable excipients and/or additives may be used in the formulations of the invention. Commonly used excipients/additives, such as pharmaceutically acceptable chelators (for example, but not limited to, EDTA, DTPA or EGTA) can optionally be added to the formulations of the invention to reduce aggregation. These additives are particularly useful if a pump or plastic container is used to administer the formulation.

Preservatives, such as phenol, m-cresol, p-cresol, o-cresol, chlorocresol, benzyl alcohol, phenylmercuric nitrite, phenoxyethanol, formaldehyde, chlorobutanol, magnesium chloride (for example, but not limited to, hexahydrate), alkylparaben (methyl, ethyl, propyl, butyl and the like), benzalkonium chloride, benzethonium chloride, sodium dehydroacetate and thimerosal, or mixtures thereof can optionally be added to the formulations of the invention at any suitable concentration such as between about 0.001% to about 5%, or any range or value therein. The concentration of preservative used in the formulations of the invention is a concentration sufficient to yield a microbial effect. Such concentrations are dependent on the preservative selected and are readily determined by the skilled artisan.

Other contemplated excipients/additives, which may be utilized in the formulations of the invention include, for example, flavoring agents, antimicrobial agents, sweeteners, antioxidants, antistatic agents, lipids such as phospholipids or fatty acids, steroids such as cholesterol, protein excipients such as serum albumin (human serum albumin (HSA), recombinant human albumin (rHA), gelatin, casein, salt-forming counterions such as sodium and the like. These and additional known pharmaceutical excipients and/or additives suitable for use in the formulations of the invention are known in the art, *e.g.*, as listed in "Remington: The Science & Practice of Pharmacy", 21st ed., Lippincott Williams & Wilkins, (2005), and in the "Physician's Desk Reference", 60th ed., Medical Economics, Montvale, N.J. (2005). Pharmaceutically acceptable carriers can be routinely selected that are suitable for the mode

of administration, solubility and/or stability of protein with a modulated glycosylation profile (*e.g.*, an antibody or DVD-Ig), as well known those in the art or as described herein.

In one embodiment, the compositions comprising a protein with a modulated glycosylation profile of the invention are formulated with the same or similar excipients and buffers as are present in the commercial adalimumab (HUMIRA[®]) formulation, as described in the "Highlights of Prescribing Information" for HUMIRA[®] (adalimumab) Injection (Revised Jan. 2008) the contents of which are hereby incorporated herein by reference. For example, each prefilled syringe of HUMIRA[®], which is administered subcutaneously, delivers 0.8 mL (40 mg) of drug product to the subject. Each 0.8 mL of HUMIRA[®] contains 40 mg adalimumab, 4.93 mg sodium chloride, 0.69 mg monobasic sodium phosphate dihydrate, 1.22 mg dibasic sodium phosphate dihydrate, 0.24 mg sodium citrate, 1.04 mg citric acid monohydrate, 9.6 mg mannitol, 0.8 mg polysorbate 80, and water for Injection, USP. Sodium hydroxide is added as necessary to adjust pH.

It will be understood by one skilled in the art that the formulations of the compositions comprising a protein with a modulated glycosylation profile of the invention may be isotonic with human blood, wherein the formulations of the invention have essentially the same osmotic pressure as human blood. Such isotonic formulations will generally have an osmotic pressure from about 250 mOSm to about 350 mOSm. Isotonicity can be measured by, for example, using a vapor pressure or ice-freezing type osmometer. Tonicity of a formulation is adjusted by the use of tonicity modifiers. "Tonicity modifiers" are those pharmaceutically acceptable inert substances that can be added to the formulation to provide an isotonicity of the formulation. Tonicity modifiers suitable for this invention include, but are not limited to, saccharides, salts and amino acids.

In certain embodiments, the formulations of the compositions comprising a protein with a modulated glycosylation profile of the invention have an osmotic pressure from about 100 mOSm to about 1200 mOSm, or from about 200 mOSm to about 1000 mOSm, or from about 200 mOSm to about 800 mOSm, or from about 200 mOSm to about 600 mOSm, or from about 250 mOSm to about 500 mOSm, or from about 250 mOSm to about 400 mOSm, or from about 250 mOSm to about 350 mOSm.

The concentration of any one component or any combination of various components, of the formulations of the compositions comprising a protein with a modulated glycosylation profile of the invention is adjusted to achieve the desired tonicity of the final formulation. For example, the ratio of the carbohydrate excipient to protein with a modulated glycosylation profile (*e.g.*, antibody or DVD-Ig) may be adjusted according to methods

known in the art (*e.g.*, U.S. Patent No. 6,685,940). In certain embodiments, the molar ratio of the carbohydrate excipient to protein with a modulated glycosylation profile (*e.g.*, antibody or DVD-Ig) may be from about 100 moles to about 1000 moles of carbohydrate excipient to about 1 mole of protein with a modulated glycosylation profile, or from about 200 moles to about 6000 moles of carbohydrate excipient to about 1 mole of protein with a modulated glycosylation profile, or from about 100 moles to about 510 moles of carbohydrate excipient to about 1 mole of protein with a modulated glycosylation profile, or from about 100 moles to about 600 moles of carbohydrate excipient to about 1 mole of protein with a modulated glycosylation profile.

The desired isotonicity of the final formulation may also be achieved by adjusting the salt concentration of the formulations. Pharmaceutically acceptable salts and those suitable for this invention as tonicity modifiers include, but are not limited to, sodium chloride, sodium succinate, sodium sulfate, potassium chloride, magnesium chloride, magnesium sulfate, and calcium chloride. In specific embodiments, formulations of the invention comprise NaCl, MgCl₂, and/or CaCl₂. In one embodiment, concentration of NaCl is between about 75 mM and about 150 mM. In another embodiment, concentration of MgCl₂ is between about 1 mM and about 100 mM. Pharmaceutically acceptable amino acids including those suitable for this invention as tonicity modifiers include, but are not limited to, proline, alanine, L-arginine, asparagine, L-aspartic acid, glycine, serine, lysine, and histidine.

In one embodiment the formulations of the compositions comprising a protein with a modulated glycosylation profile of the invention are pyrogen-free formulations which are substantially free of endotoxins and/or related pyrogenic substances. Endotoxins include toxins that are confined inside a microorganism and are released only when the microorganisms are broken down or die. Pyrogenic substances also include fever-inducing, thermostable substances (glycoproteins) from the outer membrane of bacteria and other microorganisms. Both of these substances can cause fever, hypotension and shock if administered to humans. Due to the potential harmful effects, even low amounts of endotoxins must be removed from intravenously administered pharmaceutical drug solutions. The Food & Drug Administration ("FDA") has set an upper limit of 5 endotoxin units (EU) per dose per kilogram body weight in a single one hour period for intravenous drug applications (The United States Pharmacopeial Convention, Pharmacopeial Forum 26 (1):223 (2000)). When therapeutic proteins are administered in amounts of several hundred or thousand milligrams per kilogram body weight, as can be the case with proteins of interest (*e.g.*, antibodies), even trace amounts of harmful and dangerous endotoxin must be removed.

In certain specific embodiments, the endotoxin and pyrogen levels in the composition are less than 10 EU/mg, or less than 5 EU/mg, or less than 1 EU/mg, or less than 0.1 EU/mg, or less than 0.01 EU/mg, or less than 0.001 EU/mg.

When used for *in vivo* administration, the formulations of the compositions comprising a protein with a modulated glycosylation profile of the invention should be sterile. The formulations of the invention may be sterilized by various sterilization methods, including sterile filtration, radiation, etc. In one embodiment, the protein with a modulated glycosylation profile (*e.g.*, antibody or DVD-Ig) formulation is filter-sterilized with a presterilized 0.22-micron filter. Sterile compositions for injection can be formulated according to conventional pharmaceutical practice as described in "Remington: The Science & Practice of Pharmacy", 21st ed., Lippincott Williams & Wilkins, (2005). Formulations comprising proteins of interest (*e.g.*, antibody or DVD-Ig.), such as those disclosed herein, ordinarily will be stored in lyophilized form or in solution. It is contemplated that sterile compositions comprising proteins of interest (*e.g.*, antibody or DVD-Ig) are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having an adapter that allows retrieval of the formulation, such as a stopper pierceable by a hypodermic injection needle. In one embodiment, a composition of the invention is provided as a pre-filled syringe.

In one embodiment, a formulation of the compositions comprising a protein with a modulated glycosylation profile of the invention is a lyophilized formulation. The term "lyophilized" or "freeze-dried" includes a state of a substance that has been subjected to a drying procedure such as lyophilization, where at least 50% of moisture has been removed.

The phrase "bulking agent" includes a compound that is pharmaceutically acceptable and that adds bulk to a lyo cake. Bulking agents known to the art include, for example, carbohydrates, including simple sugars such as dextrose, ribose, fructose and the like, alcohol sugars such as mannitol, inositol and sorbitol, disaccharides including trehalose, sucrose and lactose, naturally occurring polymers such as starch, dextrans, chitosan, hyaluronate, proteins (*e.g.*, gelatin and serum albumin), glycogen, and synthetic monomers and polymers.

A "lyoprotectant" is a molecule which, when combined with a protein with a modulated glycosylation profile (such as an antibody or DVD-Ig of the invention), significantly prevents or reduces chemical and/or physical instability of the protein upon lyophilization and subsequent storage. Lyoprotectants include, but are not limited to, sugars and their corresponding sugar alcohols; an amino acid such as monosodium glutamate or histidine; a methylamine such as betaine; a lyotropic salt such as magnesium sulfate; a polyol

such as trihydric or higher molecular weight sugar alcohols, *e.g.*, glycerin, dextran, erythritol, glycerol, arabitol, xylitol, sorbitol, and mannitol; propylene glycol; polyethylene glycol; PLURONICS™; and combinations thereof. Additional examples of lyoprotectants include, but are not limited to, glycerin and gelatin, and the sugars mellibiose, melezitose, raffinose, mannatriose and stachyose. Examples of reducing sugars include, but are not limited to, glucose, maltose, lactose, maltulose, iso-maltulose and lactulose. Examples of non-reducing sugars include, but are not limited to, non-reducing glycosides of polyhydroxy compounds selected from sugar alcohols and other straight chain polyalcohols. Examples of sugar alcohols include, but are not limited to, monoglycosides, compounds obtained by reduction of disaccharides such as lactose, maltose, lactulose and maltulose. The glycosidic side group can be either glucosidic or galactosidic. Additional examples of sugar alcohols include, but are not limited to, glucitol, maltitol, lactitol and iso-maltulose. In specific embodiments, trehalose or sucrose is used as a lyoprotectant.

The lyoprotectant is added to the pre-lyophilized formulation in a "lyoprotecting amount" which means that, following lyophilization of the protein in the presence of the lyoprotecting amount of the lyoprotectant, the protein essentially retains its physical and chemical stability and integrity upon lyophilization and storage.

In one embodiment, the molar ratio of a lyoprotectant (*e.g.*, trehalose) and protein with a modulated glycosylation profile (*e.g.*, antibody or DVD-Ig) molecules of a formulation of the invention is at least about 10, at least about 50, at least about 100, at least about 200, or at least about 300. In another embodiment, the molar ratio of a lyoprotectant (*e.g.*, trehalose) and protein with a modulated glycosylation profile molecules of a formulation of the invention is about 1, is about 2, is about 5, is about 10, about 50, about 100, about 200, or about 300.

A "reconstituted" formulation is one which has been prepared by dissolving a lyophilized protein with a modulated glycosylation profile (*e.g.*, antibody or DVD-Ig) formulation in a diluent such that the protein with a modulated glycosylation profile is dispersed in the reconstituted formulation. The reconstituted formulation is suitable for administration (*e.g.*, parenteral administration) to a patient to be treated with the protein with a modulated glycosylation profile and, in certain embodiments of the invention, may be one which is suitable for intravenous administration.

The "diluent" of interest herein is one which is pharmaceutically acceptable (safe and non-toxic for administration to a human) and is useful for the preparation of a liquid formulation, such as a formulation reconstituted after lyophilization. In some embodiments,

diluents include, but are not limited to, sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (*e.g.*, phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution. In an alternative embodiment, diluents can include aqueous solutions of salts and/or buffers.

5 In certain embodiments, a formulation of the compositions comprising a protein with a modulated glycosylation profile of the invention is a lyophilized formulation comprising a protein with a modulated glycosylation profile (*e.g.*, antibody or DVD-Ig) of the invention, wherein at least about 90%, at least about 95%, at least about 97%, at least about 98%, or at least about 99% of the protein with a modulated glycosylation profile may be recovered from
10 a vial upon shaking the vial for 4 hours at a speed of 400 shakes per minute wherein the vial is filled to half of its volume with the formulation. In another embodiment, a formulation of the invention is a lyophilized formulation comprising a protein with a modulated glycosylation profile of the invention, wherein at least about 90%, at least about 95%, at least about 97%, at least about 98%, or at least about 99% of the protein with a modulated
15 glycosylation profile may be recovered from a vial upon subjecting the formulation to three freeze/thaw cycles wherein the vial is filled to half of its volume with the formulation. In a further embodiment, a formulation of the invention is a lyophilized formulation comprising a protein with a modulated glycosylation profile of the invention, wherein at least about 90%, at least about 95%, at least about 97%, at least about 98%, or at least about 99% of the
20 protein with a modulated glycosylation profile may be recovered by reconstituting a lyophilized cake generated from the formulation.

In one embodiment, a reconstituted liquid formulation may comprise a protein with a modulated glycosylation profile (*e.g.*, antibody or DVD-Ig) of the invention at the same concentration as the pre-lyophilized liquid formulation.

25 In another embodiment, a reconstituted liquid formulation may comprise a protein with a modulated glycosylation profile (*e.g.*, antibody or DVD-Ig) of the invention at a higher concentration than the pre-lyophilized liquid formulation, *e.g.*, about 2 fold, about 3 fold, about 4 fold, about 5 fold, about 6 fold, about 7 fold, about 8 fold, about 9 fold, or about 10 fold higher concentration of a protein with a modulated glycosylation profile than the pre-
30 lyophilized liquid formulation.

In yet another embodiment, a reconstituted liquid formulation may comprise a protein with a modulated glycosylation profile (*e.g.*, antibody or DVD-Ig) of the invention at a lower concentration than the pre-lyophilized liquid formulation, *e.g.*, about 2 fold, about 3 fold, about 4 fold, about 5 fold, about 6 fold, about 7 fold, about 8 fold, about 9 fold or about 10

fold lower concentration of a protein with a modulated glycosylation profile than the pre-lyophilized liquid formulation.

The pharmaceutical formulations of the compositions comprising a protein with a modulated glycosylation profile, of the invention are typically stable formulations, *e.g.*, stable at room temperature.

The terms “stability” and “stable” as used herein in the context of a formulation comprising a protein with a modulated glycosylation profile (*e.g.*, an antibody or DVD-Ig) of the invention refer to the resistance of the protein in the formulation to aggregation, degradation or fragmentation under given manufacture, preparation, transportation and storage conditions. The “stable” formulations of the invention retain biological activity under given manufacture, preparation, transportation and storage conditions. The stability of the protein with a modulated glycosylation profile can be assessed by degrees of aggregation, degradation or fragmentation, as measured by HPSEC, static light scattering (SLS), Fourier Transform Infrared Spectroscopy (FTIR), circular dichroism (CD), urea unfolding techniques, intrinsic tryptophan fluorescence, differential scanning calorimetry, and/or ANS binding techniques, compared to a reference formulation. For example, a reference formulation may be a reference standard frozen at -70°C consisting of 10 mg/ml of a protein with a modulated glycosylation profile of the invention in PBS.

Therapeutic formulations of the compositions comprising a protein with a modulated glycosylation profile of the invention may be formulated for a particular dosage. Dosage regimens may be adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the protein with a modulated glycosylation profile (*e.g.*, antibody or DVD-Ig) of the invention, and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such a protein with a modulated glycosylation profile for the treatment of sensitivity in individuals.

Therapeutic compositions of the compositions comprising a protein with a modulated glycosylation profile of the invention, can be formulated for particular routes of administration, such as oral, nasal, pulmonary, topical (including buccal and sublingual), rectal, vaginal and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the subject being treated, and the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the composition which produces a therapeutic effect. By way of example, in certain embodiments, the proteins with modulated glycosylation profiles (including fragments of the protein with a modulated glycosylation profile) are formulated for intravenous administration. In certain other embodiments, the proteins with modulated glycosylation profiles (*e.g.*, antibody or DVD-Ig), of the invention, including fragments of the proteins with modulated glycosylation profiles (*e.g.*, antibody fragments) of the invention, are formulated for local delivery to the cardiovascular system, for example, via catheter, stent, wire, intramyocardial delivery, intrapericardial delivery, or intraendocardial delivery.

Formulations of the compositions comprising a protein with a modulated glycosylation profile of the invention, which are suitable for topical or transdermal administration include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants which may be required (US Patent No. 7,378,110; 7,258,873; 7,135,180; 7,923,029; and US Publication No. 20040042972).

The phrases “parenteral administration” and “administered parenterally” as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of the compositions comprising a protein with a modulated glycosylation profile of the invention, may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of

administration, without being toxic to the patient. The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

In certain embodiments, the proteins with modulated glycosylation profiles (*e.g.*, antibody or DVD-Ig) of the invention can be formulated to ensure proper distribution *in vivo*. For example, the blood-brain barrier (BBB) excludes many highly hydrophilic compounds. To ensure that the therapeutic compounds of the invention can cross the BBB (if desired), they can be formulated, for example, in liposomes. For methods of manufacturing liposomes, see, *e.g.*, U.S. Pat. Nos. 4,522,811; 5,374,548; 5,399,331. The liposomes may comprise one or more moieties which are selectively transported into specific cells or organs, thus enhance targeted drug delivery (see, *e.g.*, V. V. Ranade (1989) *J. Clin. Pharmacol.* 29:685). Exemplary targeting moieties include folate or biotin (see, *e.g.*, U.S. Pat. No. 5,416,016); mannosides (Umezawa *et al.*, (1988) *Biochem. Biophys. Res. Commun.* 153:1038); antibodies (P. G. Bloeman *et al.* (1995) *FEBS Lett.* 357:140; M. Owais *et al.* (1995) *Antimicrob. Agents Chemother.* 39:180); surfactant Protein A receptor (Briscoe *et al.* (1995) *Am. J. Physiol.* 269:9090); see also K. Keinänen; M. L. Laukkanen (1994) *FEBS Lett.* 346:123; J. J. Killian; I. J. Fidler (1994) *Immunomethods* 4:273. In one embodiment of the invention, the therapeutic compounds of the invention are formulated in liposomes; in another embodiment, the liposomes include a targeting moiety. In another embodiment, the therapeutic compounds in the liposomes are delivered by bolus injection to a site proximal to the desired area. When administered in this manner, the composition must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and may be preserved against the contaminating action of microorganisms such as bacteria and fungi. Additionally or alternatively, the proteins with modulated glycosylation profiles (*e.g.*, antibodies or DVD-Igs) of the invention may be delivered locally to the brain to mitigate the risk that the blood brain barrier slows effective delivery.

In certain embodiments, the compositions comprising a protein with a modulated glycosylation profile of the invention may be administered with medical devices known in the art. For example, in certain embodiments a protein with a modulated glycosylation profile (*e.g.*, antibody or DVD-Ig) or a fragment of protein with a modulated glycosylation profile (*e.g.*, antibody fragment) is administered locally via a catheter, stent, wire, or the like. For example, in one embodiment, a therapeutic composition of the invention can be administered with a needleless hypodermic injection device, such as the devices disclosed in U.S. Pat. Nos. 5,399,163; 5,383,851; 5,312,335; 5,064,413; 4,941,880; 4,790,824; 4,596,556. Examples of well-known implants and modules useful in the present invention include: U.S. Pat. No. 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; U.S. Pat. No. 4,486,194, which discloses a therapeutic device for administering medicants through the skin; U.S. Pat. No. 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion rate; U.S. Pat. No. 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; U.S. Pat. No. 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments; and U.S. Pat. No. 4,475,196, which discloses an osmotic drug delivery system. Many other such implants, delivery systems, and modules are known to those skilled in the art.

The efficient dosages and the dosage regimens for the compositions comprising a protein with a modulated glycosylation profile of the invention depend on the disease or condition to be treated and can be determined by the persons skilled in the art. One of ordinary skill in the art would be able to determine such amounts based on such factors as the subject's size, the severity of the subject's symptoms, and the particular composition or route of administration selected.

VI. Kits and Articles of Manufacture Comprising the Compositions Comprising Proteins with Modulated Glycosylation Profiles of the Invention

Also within the scope of the present invention are kits comprising the compositions comprising a protein with a modulated glycosylation profile, for example a protein such as an antibody, antigen-binding portion thereof, or a DVD-Ig, with a decreased fucosylation level or amount and/or an increased mannosylation level or amount of the invention and instructions for use. The term "kit" as used herein refers to a packaged product comprising components with which to administer the protein with a modulated glycosylation profile

(*e.g.*, antibody, or antigen-binding portion thereof, or DVD-Ig), of the invention for treatment of a disease or disorder. The kit may comprise a box or container that holds the components of the kit. The box or container is affixed with a label or a Food and Drug Administration approved protocol. The box or container holds components of the invention which may be
5 contained within plastic, polyethylene, polypropylene, ethylene, or propylene vessels. The vessels can be capped-tubes or bottles. The kit can also include instructions for administering a protein with a modulated glycosylation profile (*e.g.*, an antibody or a DVD-Ig) of the invention.

The kit can further contain one more additional reagents, such as an
10 immunosuppressive reagent, a cytotoxic agent or a radiotoxic agent or one or more additional proteins of interest of the invention (*e.g.*, an antibody having a complementary activity which binds to an epitope in the TNF α antigen distinct from a first anti-TNF α antibody). Kits typically include a label indicating the intended use of the contents of the kit. The term label includes any writing, or recorded material supplied on or with the kit, or which otherwise
15 accompanies the kit.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with a liquid formulation or lyophilized formulation of a protein with a modulated glycosylation profile (*e.g.*, an antibody, or antibody fragment thereof, or a DVD-Ig) of the invention. In one embodiment, a container filled with a liquid formulation of the
20 invention is a pre-filled syringe. In a specific embodiment, the formulations of the invention are formulated in single dose vials as a sterile liquid. For example, the formulations may be supplied in 3 cc USP Type I borosilicate amber vials (West Pharmaceutical Services - Part No. 6800-0675) with a target volume of 1.2 mL. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the
25 manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

In one embodiment, a container filled with a liquid formulation of the invention is a pre-filled syringe. Any pre-filled syringe known to one of skill in the art may be used in combination with a liquid formulation of the invention. Pre-filled syringes that may be used
30 are described in, for example, but not limited to, PCT Publications WO05032627, WO08094984, WO9945985, WO03077976, US Patents US6792743, US5607400, US5893842, US7081107, US7041087, US5989227, US6807797, US6142976, US5899889, US7699811, US7540382, US7998120, US7645267, and US Patent Publication No.

US20050075611. Pre-filled syringes may be made of various materials. In one embodiment a pre-filled syringe is a glass syringe. In another embodiment a pre-filled syringe is a plastic syringe. One of skill in the art understands that the nature and/or quality of the materials used for manufacturing the syringe may influence the stability of a protein formulation stored in the syringe. For example, it is understood that silicon based lubricants deposited on the inside surface of the syringe chamber may affect particle formation in the protein formulation. In one embodiment, a pre-filled syringe comprises a silicone based lubricant. In one embodiment, a pre-filled syringe comprises baked on silicone. In another embodiment, a pre-filled syringe is free from silicone based lubricants. One of skill in the art also understands that small amounts of contaminating elements leaching into the formulation from the syringe barrel, syringe tip cap, plunger or stopper may also influence stability of the formulation. For example, it is understood that tungsten introduced during the manufacturing process may adversely affect formulation stability. In one embodiment, a pre-filled syringe may comprise tungsten at a level above 500 ppb. In another embodiment, a pre-filled syringe is a low tungsten syringe. In another embodiment, a pre-filled syringe may comprise tungsten at a level between about 500 ppb and about 10 ppb, between about 400 ppb and about 10 ppb, between about 300 ppb and about 10 ppb, between about 200 ppb and about 10 ppb, between about 100 ppb and about 10 ppb, between about 50 ppb and about 10 ppb, between about 25 ppb and about 10 ppb.

In certain embodiments, kits comprising a protein with a modulated glycosylation profile such as a decreased fucosylation level or amount and/or an increased mannosylation level or amount (*e.g.*, an antibody or DVD-Ig) of the invention are also provided that are useful for various purposes, *e.g.*, research and diagnostic including for purification or immunoprecipitation of a protein with a modulated glycosylation profile from cells, detection of the protein with a modulated glycosylation profile *in vitro* or *in vivo*. For isolation and purification of a protein with a modulated glycosylation profile, the kit may contain an antibody coupled to beads (*e.g.*, sepharose beads). Kits may be provided which contain the antibodies for detection and quantitation of a protein with a modulated glycosylation profile *in vitro*, *e.g.*, in an ELISA or a Western blot. As with the article of manufacture, the kit comprises a container and a label or package insert on or associated with the container. The container holds a composition comprising at least one protein with a modulated glycosylation profile (*e.g.*, antibody or DVD-Ig) of the invention. Additional containers may be included that contain, *e.g.*, diluents and buffers, control proteins with modulated glycosylation profiles

(*e.g.*, antibody or DVD-Ig). The label or package insert may provide a description of the composition as well as instructions for the intended *in vitro* or diagnostic use.

The present invention also encompasses a finished packaged and labeled pharmaceutical product. This article of manufacture includes the appropriate unit dosage form in an appropriate vessel or container such as a glass vial, pre-filled syringe or other container that is hermetically sealed. In one embodiment, the unit dosage form is provided as a sterile particulate free solution comprising a protein with a modulated glycosylation profile (*e.g.*, an antibody or DVD-Ig) that is suitable for parenteral administration. In another embodiment, the unit dosage form is provided as a sterile lyophilized powder comprising a protein with a modulated glycosylation profile (*e.g.*, an antibody or DVD-Ig) that is suitable for reconstitution.

In one embodiment, the unit dosage form is suitable for intravenous, intramuscular, intranasal, oral, topical or subcutaneous delivery. Thus, the invention encompasses sterile solutions suitable for each delivery route. The invention further encompasses sterile lyophilized powders that are suitable for reconstitution.

As with any pharmaceutical product, the packaging material and container are designed to protect the stability of the product during storage and shipment. Further, the products of the invention include instructions for use or other informational material that advise the physician, technician or patient on how to appropriately prevent or treat the disease or disorder in question, as well as how and how frequently to administer the pharmaceutical. In other words, the article of manufacture includes instruction means indicating or suggesting a dosing regimen including, but not limited to, actual doses, monitoring procedures, and other monitoring information.

Specifically, the invention provides an article of manufacture comprising packaging material, such as a box, bottle, tube, vial, container, pre-filled syringe, sprayer, insufflator, intravenous (i.v.) bag, envelope and the like; and at least one unit dosage form of a pharmaceutical agent contained within the packaging material, wherein the pharmaceutical agent comprises a liquid formulation containing a protein with a modulated glycosylation profile (*e.g.*, an antibody or DVD-Ig). The packaging material includes instruction means which indicate how that the protein with a modulated glycosylation profile (*e.g.*, antibody or DVD-Ig) can be used to prevent, treat and/or manage one or more symptoms associated with a disease or disorder.

The present invention is further illustrated by the following examples which should not be construed as limiting.

EXAMPLES

5 Example 1:

1. Materials & Methods

Cell Culture

Two recombinant Chinese Hamster Ovary (CHO) cell lines expressing two different humanized monoclonal antibodies (Antibody 1, Antibody 2) were evaluated in two different culture vessels (shaker flasks and laboratory scale bioreactors). Both cell lines were of CHO DUX-B11 origin based on a dhfr (dihydrofolate reductase) expression system. Both cell lines were cultured in the same chemically defined basal media (CDBM), with Cell Line 1 also utilizing a chemically-defined feed media (CDFM). Each of the respective media were supplemented with selected monosaccharides and oligosaccharides to evaluate their potential impact on the resulting N-glycan oligosaccharide profile. In preparing the cultures, the cell lines were serially expanded through separate seed train inoculums to generate enough cells for inoculation. Process conditions utilized during the cultures were slightly different between the two different cell lines as shown in Table 1, but similar between each cell line and the respective non-sugar supplemented control conditions.

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Table 1 : Summary of cell culture process conditions & sugar supplementation details

	Cell Line 1			Cell Line 2
Culture Vessel	250 mL shaker flask	250 mL shaker flask	3L lab-scale bioreactors	250 mL shaker flask
Culture Mode	Fedbatch	Fedbatch	Fedbatch	Semi-Batch ^a
Initial Culture Temperature (°C)	36	36	36	36
Dissolved Oxygen (%)	N/A ^b	N/A ^b	30	N/A ^b
pH	N/A ^b	N/A ^b	6.9	N/A ^b
Sugar Supplements Evaluated ^c	Sucrose	Tagatose	Sucrose Tagatose	Sucrose
Supplement Concentrations (mM)	0, 30, 50, 70	0, 30, 50, 70	0, 50	0, 7, 15, 30

^a No feed media utilized; concentrated glucose solution addition utilized when glucose levels dropped below 3 g/L.

^b Cultures run in CO₂ incubators at 5% CO₂ in air; pH and DO parameters were not controlled, and thus did not have setpoint values.

^c Supplements added to both chemically-defined basal & feed media (when used).

Viable cell density (VCD) and cell viability values were measured through trypan blue exclusion via Cedex automated cell counters (Roche Applied Science, Indianapolis, IN), glucose and lactate values were measured with a ABL-805 (Radiometer Medical, Denmark) blood gas analyzer. Offline pH, dissolved oxygen (DO), and pCO₂ measurements were performed with a ABL-805 (Radiometer Medical, Denmark) blood gas analyzer. Osmolality was measured on a Multi-Osmette 2430 osmometer (Precision Systems, Natick, MA).

Protein A Affinity Chromatography

Antibody titers were measured from crude cell culture harvests on a Poros A™ (Life Technologies, Carlsbad, CA) affinity column using an Agilent (Santa Clara, CA) 1200 Series HPLC, or equivalent, operating with a low pH, step elution gradient with detection at 280 nm. Absolute concentrations were assigned with respect to reference standard calibration curves.

Purified antibodies subjected to additional analytical characterization were purified using MabSelect™ Protein A (GE Healthcare, Piscataway, NJ) using a low pH, step elution gradient, followed by buffer exchange using Corning Lifesciences (Tewksbury, MA) Spin Concentrator X UF columns according to the manufacturers' recommended procedures.

N-glycan Oligosaccharide Profiling

Approximately 200 µg of Protein A purified samples from Cell Lines 1 and 2 were treated with N-glycanase at 37°C overnight to remove the N-glycans from the protein. The protein was precipitated and the supernatant was taken for subsequent chemical derivatization of the reducing end of the released glycans with 2-aminobenzamide (2-AB) dye. Following the derivatization step, the excess label was removed using clean up cartridges and the samples were analyzed using normal phase HPLC with fluorescent detection. Mobile phase A was 100% acetonitrile and mobile phase B was 50 mM ammonium formate pH 4.4. The glycans were eluted from a polyamide column (Prozyme, Hayward, CA) using a shallow gradient. The labeled glycans were detected using a fluorescence detector with an excitation wavelength of 330 nm and an emission wavelength of 420 nm.

2. Results

Shake Flask Screening of Select Monosaccharides and Oligosaccharides

Cell Line 1 was cultured in shake flasks in fedbatch mode after an abbreviated seed train. Sucrose and tagatose were supplemented into chemically-defined basal and feed media at concentrations of 30, 50, and 70 mM, and compared to an unsupplemented control condition. The viable cell density (VCD), viability, and harvest titer results are shown in Figures 3A-3C and Figures 5A-5C for the sucrose, and tagatose conditions, respectively. Both sugars facilitated a slight decrease in VCD, with the higher concentrations also supporting the lowest peak VCD. Viability results were however more comparable to the control, unsupplemented conditions, with the exception of the 70 mM sucrose condition, which resulted in a decrease in cell viability that was larger, and earlier in the culture,

compared to control conditions. Harvest titer results demonstrated that 30 mM and 50 mM sucrose did not adversely impact titer, but 70 mM sucrose did reduce the harvest titer significantly. 30 mM tagatose did not adversely impact harvest titer, but 50 mM and 70 mM tagatose did. Collectively, these results indicate that at certain concentrations of sucrose (e.g., 30 mM and 50 mM) and tagatose (e.g., 30 mM) there is a decrease in viable cell density, however percent cell viability and harvest titer profile remain comparable to unsupplemented culture conditions. As such, the upper threshold of sucrose or tagatose concentration should not be exceeded.

There was a significant impact on the overall N-glycan distribution when cell cultures were supplemented with sucrose (Figure 4). A concentration-dependent response was observed across all sucrose concentrations evaluated. Notably there was a 24% increase in Man 5 species, 12% increase in Man 6 species, and a 7% increase in Man 7 species at the 70 mM sucrose supplementation condition. This cumulative increase of 43% in overall mannosylated N-glycans was accompanied by a 51% drop in NGA2F species, the majority of which was precluded from being formed due to the abrogation of the N-glycan biosynthetic reaction as shown in Figure 2. The data also demonstrate that combining the 51% decrease in NGA2F species and the 7% increase in NGA2F-GlcNAc species results in an overall decrease in the fucosylation level of 44%. This significant drop in fucosylation is important as it has been shown that decreased fucosylation of N-glycans has a significant and positive effect on the overall level of ADCC response. These data demonstrate that the supplementation of sucrose into cell culture media is capable of significantly re-distributing the overall protein glycosylation profile of the produced protein, and for some commercial protein therapeutics, this is a desired product quality attribute.

There was also a significant impact on the overall N-glycan distribution when the cell culture media was supplemented with tagatose (Figure 6). Similar to the sucrose supplemented cell culture conditions, there was a concentration-dependent response across all of the concentrations evaluated. The results indicate that there was at most a 10% increase in Man 5 species, 6% increase in Man 6 species, 3% increase in Man 7 species, and a 1% increase in Man 8 species at the 70 mM tagatose supplementation condition. This cumulative 20% increase in overall mannosylated N-glycans was accompanied by a 30% decrease in NGA2F species. In addition, there was an overall 23% decrease in overall fucosylation levels. Comparison of the results of sucrose and tagatose supplementation demonstrate that the percent increase of N-glycans was lower in the tagatose supplemented cultures as

compared to the sucrose supplemented cultures. However, the individual N-glycan species which were modulated, were similar between the sucrose and tagatose supplemented cultures. Both sugars utilize the same mechanism to modulate the protein N-glycosylation profile as the pattern of modulation of the individual N-glycans species was consistent between both sugars.

Laboratory-scale Bioreactor Confirmation of the Targeted Modulation of Protein Glycosylation Profiles

3L scale-down model bioreactors were utilized to verify the impact of select sugars on the resulting protein glycosylation profiles. The concentration of the sucrose and tagatose was chosen to minimize any potential adverse impact on cell growth and productivity, but still facilitate a measurable modulation of the glycosylation profile. Cell culture process performance indicators were monitored and measured throughout the respective cultures. Viable cell density, cell viability, lactate, pCO₂, osmolality, harvest titer, and harvest N-glycan oligosaccharide data were measured and reported.

Figures 7A-7F highlight the cell culture performance results observed through the use of sucrose supplementation in the basal and feed medias. In laboratory-scale bioreactors there was no impact on viable cell density or cell viability upon supplementation with 50 mM sucrose when compared to the unsupplemented control condition. Dissolved CO₂ (pCO₂) and lactate production are direct measures of the respiratory and metabolic activities of mammalian cells, respectively. There was no significant difference in pCO₂ between the sucrose supplemented and non-supplemented cultures, indicating no net change in the overall respiratory activity of the cells. With respect to lactate, there was only a nominal increase in overall levels throughout the sucrose supplemented culture, which is consistent with the overall higher level of residual sugar in the culture media. Osmolality of the sucrose supplemented condition was only slightly higher compared to the control mostly due to the additional sugar solute. Upon harvest of the cultures, the final titer ratio of the sucrose supplemented culture was 0.78 suggesting a slight drop in overall antibody productivity with inclusion of 50 mM sucrose. These data are consistent with the results observed from the shake flask cultures. After Protein A purification, the N-glycan glycoform profile was measured (Figure 8). Similar to the shake flask results, there was a significant increase in overall mannosylation levels; Man 7 increased 3%, Man 6 increased 5%, and Man 5 increased 16%. This 24% increase in overall mannosylation was accompanied by a 29%

decrease in NGA2F species, and an overall 24% decrease in fucosylation. These changes are very significant and are consistent with the results observed in the shake flasks.

Figures 9A-9F highlight the cell culture results observed with the use of tagatose supplementation in the basal and feed medias. Tagatose had a much more pronounced effect on viable cell density as compared to the unsupplemented control. The results indicated a significant reduction in peak VCD from 11.2×10^6 cells/mL for the control unsupplemented culture versus 8.5×10^6 cells/mL for the tagatose supplemented culture. Despite this lower cell growth, cell viability remained high throughout the culture, and comparable to the results observed in the control culture. There was no significant difference in pCO_2 between the tagatose supplemented and non-supplemented cultures, suggesting no net change in the overall respiratory activity of the cells. With respect to lactate, there was only a nominal increase in overall levels throughout the tagatose supplemented culture, which is consistent with the overall higher level of residual sugar in the culture media. Osmolality of the tagatose supplemented culture was higher as compared to the control culture mostly due to the additional sugar solute. Upon harvest of the cultures, the final titer ratio of the tagatose supplemented culture was 0.90 suggesting a slight drop in overall antibody productivity upon inclusion of 50 mM tagatose. The results were consistent with the results observed from the shake flask cultures. The final harvest titer ratio of the tagatose supplemented cultures was higher than the final harvest titer ratio observed from the sucrose supplemented culture.

After Protein A purification, the N-glycan glycosylation profile was measured with the results shown in Figure 10. Similar to the shake flask results, there was a significant increase in overall mannosylation levels; Man 7 increased 1%, Man 6 increased 2%, and Man 5 increased 7%. This 10% increase in overall mannosylation was accompanied by a 15% decrease in NGA2F species, and an overall 11% decrease in fucosylation. These changes are very significant and are consistent with the results observed in the shake flasks.

The cell culture process performance results in 3L-scale bioreactors demonstrated that the observed effect on protein glycosylation in shake flasks is scale-independent. The significant increase in mannosylation, and resulting decrease in fucosylation has important implications for the optimization of the production of protein therapeutics and the modulation of protein glycosylation profiles. Indeed, the selective use of sugars such as sucrose and tagatose increases the ability of cell culture scientists to customize the product characteristics of recombinant glycoproteins expressed in mammalian cells, and the resulting therapeutic activity, efficacy, and PK.

***Evaluation of Alternative Cell Lines for the Impact of Selective Sugar
Supplementation on the Resulting Protein Glycosylation Profiles***

Cell Line 2 was evaluated in shake flask culture in semi-fedbatch mode. Sucrose was supplemented into the basal media at various concentrations to evaluate the resulting impact on cell culture performance, as well as the protein glycosylation profile. The viable cell density, viability, and harvest titer results are shown in Figures 11A-11C. On average, 7 mM, 15 mM, and 30 mM sucrose all nominally impacted overall cell growth, with only slight drops in peak viable cell density (1×10^6 - 2×10^6 cells/mL) as compared to the unsupplemented control. Cell viability and harvest titer results were not significantly different from each other. These results are consistent with the lower concentration sucrose supplementation results generated using Cell Line 1, in that at certain concentrations, there is no significant impact on overall cell growth or productivity. There was however, a very significant impact on the protein glycosylation profiles of harvest samples across all sucrose concentrations evaluated (Figure 12). A concentration-dependent modulation of protein glycosylation was observed that was consistent with the results obtained for Cell Line 1.

Notably there was up to a 17% increase in Man 5 species, 1% increase in Man 6 species, and a 1% increase in Man 7 species at the 30 mM sucrose supplementation condition. This cumulative increase of 19% in overall mannosylated N-glycans was accompanied by a 27% drop in NGA2F species, and a 19% drop in overall fucosylation. Hence, as demonstrated with Cell Line 1, there was a very significant impact on the N-glycan glycoform profile. Specifically, individual N-glycans increased or decreased with sucrose supplementation of Cell Line 2 that was consistent with the results obtained for Cell Line 1.

Example 2

1. Materials & Methods

Cell Culture

Two recombinant Chinese Hamster Ovary (CHO) cell lines expressing two different recombinant glycoproteins were evaluated in two different cultures vessels (shaker flasks and 3L laboratory scale bioreactors). Cell Line 1 expressed Antibody 1, Cell Line 2 expressed Dual Variable Domain Immunoglobulin 1 (DVD 1) and Cell Line 3 expressed Antibody 2. Antibodies 1 and 2 are IgG1 proteins, and DVD 1 is an immunoglobulin with two variable domains as documented previously (Wu C. *et al.*, (2007) *Nat. Biotechnol* 25(11):1290-1297).

All cell lines were of CHO DUX-B11 origin based on a dhfr (dihydrofolate reductase) expression system. All cell lines were cultured in the same chemically defined basal media. Cell lines 1 and 2 were fed with the same chemically-defined feed media, but Cell Line 1 was fed media that was formulated at a 50% higher concentration. Each of the respective media were supplemented with either sucrose or tagatose to evaluate their potential impact on the resulting N-glycan oligosaccharide profile. All sugars were purchased from Sigma-Aldrich (St. Louis, MO). In preparation of the cultures, the cell lines were serially expanded through separate seed train inoculums to generate enough cells for inoculation. Process conditions utilized during the cultures were slightly different between the two different cell lines as shown in Table 1, but similar between each cell line and the respective sugar supplemented control conditions.

Table 2: Summary of cell culture process conditions and sugar supplementation details

	Cell Line 1		Cell Line 2	Cell Line 3
Culture Vessel	250 mL shaker flask	3L lab-scale bioreactors	250 mL shaker flask	250 mL shaker flask
Culture Mode	Fedbatch	Fedbatch	Fedbatch	Extended Batch ^a
Initial Culture Temperature (°C)	36	36	35	36
Dissolved Oxygen (%)	N/A ^b	30	N/A ^b	N/A ^b
pH	N/A ^b	6.9	N/A ^b	N/A ^b
Sugar Supplements Evaluated ^b	Sucrose Tagatose	Sucrose Tagatose Fructose	Sucrose Tagatose	Sucrose
Supplement Concentrations (mM) ^c	0, 1, 10, 30, 50, 70	0, 50	0, 1, 30, 50	0, 7, 15, 30

^a No feed media used; glucose added to cultures as needed to preclude glucose depletion.

^bCultures run in CO₂ incubators at 5% CO₂ in air; pH and DO parameters were not controlled, and thus do not have setpoint values.

^cSupplements added to both chemically-defined basal and feed media.

5 Viable cell density (VCD) and cell viability values were measured through trypan blue exclusion via Cedex automated cell counters (Roche Applied Science, Indianapolis, IN), glucose and lactate values were measured with a ABL-805 (Radiometer Medical, Denmark) blood gas analyzer. Offline pH, dissolved oxygen (DO), and pCO₂ measurements were performed with an ABL-805 (Radiometer Medical, Denmark) blood gas analyzer.

10 Osmolality was measured on a Multi-Osmette 2430 osmometer (Precision Systems, Natick, MA).

Protein A Affinity Chromatography

Antibody titers were measured from crude cell culture harvests on a Poros ATM (Life Technologies, Carlsbad, CA) affinity column using an Agilent (Santa Clara, CA) 1200 Series HPLC, or equivalent, operating with a low pH, step elution gradient with detection at 280 nm. Absolute concentrations were assigned with respect to reference standard calibration curves.

Purified antibodies subjected to additional analytical characterization were purified using MabSelectTM Protein A (GE Healthcare, Piscataway, NJ) using a low pH, step elution gradient, followed by buffer exchange using Corning Lifesciences (Tewksbury, MA) Spin Concentrator X UF columns according to the manufacturers' recommended procedures.

N-glycan Oligosaccharide Profiling

Approximately 200 µg of Protein A purified samples from Cell Lines 1 and 2 were treated with N-glycanase at 37°C overnight to remove the N-glycans from the protein. The protein was precipitated and the supernatant was taken for subsequent chemical derivatization of the reducing end of the released glycans with 2-aminobenzamide (2-AB) dye. Following the derivatization step, the excess label was removed using clean up cartridges and the samples were analyzed using normal phase HPLC with fluorescent detection. Mobile phase A was 100% acetonitrile and mobile phase B was 50 mM ammonium formate pH 4.4. The glycans were eluted from a polyamide column (Prozyme, Hayward, CA) using a shallow

gradient. The labeled glycans were detected using a fluorescence detector with an excitation wavelength of 330 nm and an emission wavelength of 420 nm.

ADCC Measurements

ADCC activity was assessed using a ^{51}Cr release assay. Approximately 1 million
5 cells expressing the target for Antibody 2 were labeled with 50 μCi ^{51}Cr for 1 hour at 37°C, washed with culture medium, and then plated at 1×10^4 /well in a 96-well v-bottom plate. Antibody 2 was incubated with target cells for 30 minutes at 4°C before addition of PBMC effector cells. After a 4 hour incubation at 37°C, 100 μL of supernatant were collected from each well, and released radioactivity was counted with a Wallac WIZARD Gamma Counter
10 (Perkin-Elmer). All measurements were expressed as a percentage of total cell lysis.

Statistics

Experimental results are expressed as mean $\pm$ SD for those results generated from at least three independent cultures. Experimental results are expressed as the measured value for those results generated from less than three independent cultures. Results were evaluated
15 for statistical significance through 2-sided t-tests, with a requirement of $p < 0.05$ relative to the unsupplemented control conditions.

2. Results

Impact of Sucrose and Tagatose on the Protein Glycosylation Profiles of 20 ***Recombinant Antibodies***

Cell Line 1 was cultured in shake flasks in fedbatch mode after an abbreviated seed train. Sucrose and tagatose were supplemented into chemically-defined basal and feed media at concentrations of 1, 10, 30, 50, and 70 mM, and compared to unsupplemented control conditions. The viable cell density (VCD), viability, and harvest titer results are shown in
25 Figures 13A-13C for the sucrose supplemented cultures and Figures 14A-14C for the tagatose supplemented cultures.

Amongst the sucrose supplemented cultures, viable cell density remained essentially the same over time across the 1, 10, and 30 mM sucrose conditions. Only the 50 mM and 70 mM sucrose cultures demonstrated a reduction in peak VCD, which were modest changes,
30 but statistically significant. Cell viability results were similar across the various conditions up to process day 10. After process day 10, the 10, 30, 50, and 70 mM sucrose cultures all

died slower than the control, and the results were statistically significant. It is likely that the higher sugar concentration in these cultures was responsible for the prolonged cell viability. Despite these changes in VCD and viability, the Antibody 1 harvest titer results were essentially indistinguishable from the control conditions. Only the 50 mM sucrose conditions demonstrated a 2% increase in harvest titer. Thus, across the range of tested concentrations, sucrose is well tolerated by mammalian cells in culture, with only a minor impact on cell growth, and no impact on protein productivity.

The impact of sucrose on the N-glycan oligosaccharide distribution is shown in Figure 13D. There was a significant impact on the overall N-glycan distribution. A concentration-dependent response was observed across all the sucrose concentrations evaluated. Notably there was a 19% increase in Man 5 species, 10% increase in Man 6 species, 6% increase in Man 7 species, and 2% increase in Man 8 species for the 70 mM sucrose supplementation condition. This cumulative increase of 37% in overall mannosylated N-glycans was accompanied by a 44% drop in NGA2F species, the majority of which was precluded from being formed due to the abrogation of the N-glycan biosynthetic reaction shown in Figure 2. The data also demonstrate that the 44% decrease in NGA2F species, combined with a 1% decrease in NA1F species, and an 8% increase in NGA2F-GlcNAc species, resulted in an overall decrease in the fucosylation level of 37%. The 10, 30, and 50 mM sucrose cultures demonstrated similar behavior, but with lower absolute percent changes. However, the overall N-glycan core fucosylation was decreased in a statistically significant manner at 10, 30, 50 and 70 mM sucrose, which is beneficial for some recombinant proteins. The results also establish that sucrose supplementation is a new powerful method for the re-distribution of N-glycans towards high mannose glycans, which is a desirable product characteristic for some protein therapeutics.

Amongst the tagatose supplemented cultures, viable cell density remained similar or higher over time across the 1 mM and 10 mM conditions. However, the 30, 50, and 70 mM tagatose cultures demonstrated a reduction in peak VCD across many process days which was statistically significant. Similar to the sucrose supplementation results, cell viability remained high throughout each of the tagatose supplemented cultures. The lower concentrations of 1 mM and 10 mM behaved in a manner similar to the control cultures. After process day 10, the 30, 50, and 70 mM tagatose cultures all died slower than the control and the results were statistically significant across many of the days. It is likely that the higher sugar concentration in these cultures was responsible for the higher cell viability.

Despite these changes in VCD and viability, the Antibody 1 harvest titer results were essentially indistinguishable from the cells cultured under control conditions. Only the 70 mM tagatose conditions demonstrated a 9% decrease in harvest titer. Thus, across the range of tested concentrations it can be concluded that tagatose is well tolerated by mammalian cells in culture, with only a minor impact on cell growth and protein productivity.

The impact of tagatose on the N-glycan oligosaccharide distribution is shown in Figure 14D. There was a significant impact on the overall N-glycan distribution. A concentration-dependent response was observed across many of the tagatose concentrations evaluated. Notably there was an 8% increase in Man 5 species, 4% increase in Man 6 species, 2% increase in Man 7 species, and 1% increase in Man 8 species for the 70 mM tagatose supplementation condition. This cumulative increase of 15% in overall mannosylated N-glycans was accompanied by a 23% drop in NGA2F species, the majority of which was precluded from being formed due to the abrogation of the N-glycan biosynthetic reaction shown in Figure 2. The data also demonstrate that the 23% decrease in NGA2F species, combined with a 8% increase in NGA2F-GlcNAc species, resulted in an overall decrease in the fucosylation level of 15%. The 50 mM tagatose cultures demonstrated similar behavior, but with lower absolute percent changes. The 1, 10, and 30 mM tagatose cultures provided for N-glycan profiles that were not practically different compared to the control culture. The results obtained with tagatose supplementation of cell culture media are similar to the results obtained with sucrose supplementation. Supplementation of cell culture media with tagatose results in an overall decrease in core N-glycan fucosylation with potential benefits towards ADCC, and provides a novel method for the modulation of N-glycan species, as well as a method for achieving product comparability with a reference protein.

Comparison of the results of sucrose or tagatose supplementation demonstrates that the extent of change in the percent of N-glycans was lower in tagatose supplemented cultures compared to sucrose supplemented cultures. However, the specific N-glycan species which changed was similar between both conditions. Both sucrose and tagatose utilize the same mechanism for effecting the protein N-glycosylation profile since the pattern of change of the individual N-glycans was consistent between supplementation with both sugars.

Impact of Sucrose and Tagatose on the Protein Glycosylation Profiles of Dual Variable Domain Immunoglobulins

Cell Line 2 was cultured in shake flasks in fed batch mode after an abbreviated seed train. Sucrose and tagatose were supplemented into chemically-defined basal and feed media at concentrations of 1, 30, and 50 mM, and process performance results were compared to unsupplemented control conditions. The viable cell density (VCD), viability, and harvest titer results are shown in Figures 15A-15C for the sucrose supplemented cultures and Figures 16A-16C for the tagatose supplemented cultures.

Amongst the sucrose supplemented cultures, viable cell density remained approximately the same over time across the evaluated conditions. Only the 50 mM sucrose cultures demonstrated a reduction in peak VCD by at most 2×10^6 cells/mL. Cell viability results were similar across the various conditions up to process day 10. However, the relative harvest titer results were different among the conditions tested. As the sucrose concentration increased, the harvest titers decreased. The 50 mM sucrose cultures demonstrated the largest titer reduction of 48%, which was statistically significant from the control, and a much larger reduction than observed in Cell Line 1. Thus, it appears that sucrose is not as well tolerated in Cell Line 2 as compared to Cell Line 1.

The impact of sucrose on the N-glycan oligosaccharide distribution is shown in Figure 15D. There was a significant impact on the overall N-glycan distribution with sucrose supplementation. A concentration-dependent response was observed across all sucrose concentrations evaluated. Notably there was a 7% increase in Man 5 species and a 1% increase in Man 6 species with 50 mM sucrose supplementation of the culture. This cumulative increase of 8% in overall mannosylated N-glycans was accompanied by a 20% drop in NGA2F species, the majority of which was precluded from being formed due to the abrogation of the N-glycan biosynthetic reaction shown in Figure 2. The data also demonstrate that the 20% decrease in NGA2F species, combined with a 1% decrease in NA1F species, and a 8% increase in NGA2F-GlcNAc species, resulted in an overall decrease in the fucosylation level of 13%. The 30 mM sucrose cultures demonstrated a similar decrease in overall fucosylation level, however the absolute percent change was lower.

Tagatose was also evaluated with Cell Line 2 to evaluate the effect on protein glycosylation. Similar to sucrose, none of the evaluated concentrations adversely impacted the viable cell density (VCD). At all concentrations evaluated, the VCD was actually slightly

higher compared to the control. The cell viability results indicate that this parameter remained as high as the control through the majority of the process. After Day 8, the viability of the 30 mM and 50 mM tagatose supplemented cultures remained higher than the control, likely for the same reason as discussed above; the higher sugar concentration provided an enhanced level of nutrients that supported longer cell life. 1 mM tagatose did not adversely impact relative harvest titer, but both 30 mM and 50 mM tagatose significantly decreased harvest titer by 15% and 38%, respectively.

The impact of tagatose on the N-glycan oligosaccharide distribution of DVD 1 is shown in Figure 16D. There was a significant impact on the overall N-glycan distribution. A concentration-dependent response was observed across all the tagatose concentrations evaluated. Notably there was a 3% increase in Man 5 species and a 1% increase in Man 6 species for the 50 mM sucrose supplementation condition. This cumulative increase of 4% in overall mannosylated N-glycans was accompanied by a 9% decrease in NGA2F species, the majority of which was precluded from being formed due to the abrogation of the N-glycan biosynthetic reaction shown in Figure 2. The data also demonstrate that the 9% decrease in NGA2F species, combined with a 1% decrease in NA1F species, and a 5% increase in NGA2F-GlcNAc species, resulted in an overall decrease of the fucosylation level of 5%. The 30 mM tagatose cultures demonstrated a similar decrease in overall fucosylation, however the absolute percent change was lower.

Overall, sucrose and tagatose supplementation did not have as pronounced of an impact on the N-glycan profile of DVD 1 as did the supplementation on Antibody 1. The differences in the structure of these proteins contributed to the effect of sucrose or tagatose supplementation on the N-glycosylation profiles. These results demonstrate that while the absolute percentage of various N-glycan species may vary, overall, the effect of sucrose and/or tagatose supplementation on the N-glycan oligosaccharide profile is independent of the particular protein.

Impact of Sucrose and Tagatose on Protein Glycosylation Profiles is Independent of Culture Scale

3L scale laboratory bioreactors were utilized to verify the impact of sucrose, tagatose, or fructose on protein glycosylation profiles. Concentrations of the sugars were chosen to minimize any potential adverse impacts on cell growth and productivity, but still facilitate a measurable impact on the glycoform profile. Cell culture process performance indicators

were monitored and measured throughout the study. Viable cell density, cell viability, lactate, pCO₂, osmolality, harvest titer, and harvest N-glycan oligosaccharide data was measured and reported.

Figure 17 shows the cell culture performance observed through the use of sucrose, tagatose, or fructose supplementation into the basal and feed medias. In laboratory-scale bioreactors there was no impact on viable cell density (VCD) or cell viability upon supplementation with 50 mM sucrose or 50 mM fructose compared to the unsupplemented control culture. Tagatose had a much more pronounced effect on viable cell density compared to the unsupplemented control. The results indicate a significant reduction in peak VCD of 11.2×10^6 cells/mL for the control versus 8.5×10^6 cells/mL for the tagatose supplemented culture. Despite this lower cell growth, cell viability remained high throughout the culture, on par with the results observed with the control. Residual media glucose and lactate production are direct measures of the metabolic activities of mammalian cells, respectively. The three sugars evaluated, all facilitated a higher relative level of residual glucose levels compared to the unsupplemented control where glucose was the only media sugar. With respect to lactate, there was no discernible difference between the various cultures. Osmolality of the sucrose, tagatose, or fructose supplemented cultures were only slightly higher compared to the control, primarily due to the additional sugar solute. Upon harvest, the final titer ratio was 0.90 for the tagatose supplemented culture, 0.78 for the sucrose supplemented culture, and 0.81 for the fructose supplemented culture.

After Protein A purification, the N-glycan oligosaccharide profile was measured with the results provided in Figure 18. Similar to the shake flask results, there was a significant increase in overall mannosylation levels with sucrose supplementation. Man 7 increased 3%, Man 6 increased 5%, and Man 5 increased 16% for the sucrose supplemented culture. This 24% increase in overall mannosylation was accompanied by a 29% decrease in NGA2F species, 1% increase in NA1F-GlcNAc, 3% decrease in NA1F, 7% increase in NGA2F-GlcNAc, for an overall 24% decrease in fucosylation. The tagatose supplemented culture results were similar to those observed in the shake flasks. Man 7 increased 1%, Man 6 increased 2%, and Man 5 increased 7%. This 10% increase in overall mannosylation was accompanied by a 15% decrease in NGA2F species, 1% increase in NA1F-GlcNAc, 3% decrease in NA1F, and 6% increase in NGA2F-GlcNAc, for an overall 11% decrease in fucosylation. These changes in N-glycan oligosaccharide profile were consistent with the results obtained using the shake flasks.

The fructose supplemented culture did not facilitate any significant changes in protein glycosylation compared to the control. It is interesting that the results observed with sucrose supplementation, when glucose is already in the media, do not agree with the results observed with fructose supplementation into the cell culture media. Sucrose, which is comprised of glucose and fructose, modulates a protein glycosylation profile that does not correspond to the profile demonstrated when glucose and/or fructose are the predominant sugars in the cell culture. This indicates that the mechanism for sucrose modulation of the protein glycosylation profile (*e.g.*, the increase in high mannose N-glycans and decrease in the fucosylation level) is not due to its constituent fructose or glucose alone. Instead, it is the complex of glucose to fructose (*i.e.*, sucrose) that facilitates an enzymatic block at the high mannose to complex type N-glycan biosynthetic pathway (shaded areas in Figure 2).

The cell culture process performance results in 3L-scale bioreactors demonstrate that the effect of sucrose or tagatose supplementation on protein glycosylation is volumetric scale-independent. The significant increase in mannosylation, and resulting decrease in fucosylation, has important implications for the optimization of the production of protein therapeutics and the modulation of protein glycosylation profiles. Indeed, the selective use of sugars, such as sucrose and tagatose, increases the ability to customize the product characteristics of recombinant glycoproteins expressed in mammalian cells, and the resulting therapeutic activity, or comparability to a reference protein.

High Mannosylation/Low Fucosylation Effect on ADCC

Cell Line 3 was evaluated in shake flask culture in chemically defined media supplemented with 7, 15, and 30 mM sucrose. The cultures were harvested, and the N-glycan oligosaccharide profiles were measured with the results shown in Figure 20. There was a significant increase in overall mannosylation levels, and thus a significant decrease in overall fucosylation levels. The response to sucrose was dependent on the sucrose concentration and similar to the response demonstrated in Cell Lines 1 and 2. Notably there was a 17% increase in Man 5 species, 1% increase in Man 6 species, and a 1% increase in Man 7 species for the 30 mM sucrose supplementation condition. This cumulative increase of 19% in overall mannosylated N-glycans was accompanied by a 27% drop in NGA2F species, the majority of which was precluded from being formed due to the abrogation of the N-glycan biosynthetic reaction shown in Figure 2. The data also demonstrates that with the combined 27% decrease in NGA2F species and 8% increase in NGA2F-GlcNAc species, that the overall fucosylation level was decreased by 19%. Antibody 3, purified from the sucrose supplemented cell

cultures, was tested in a Cr-51 release assay to evaluate effector function, specifically ADCC activity. This was performed to verify that the lower level of fucosylated N-glycans linked to Antibody 3 would facilitate an increase in ADCC activity. The results are shown in Figure 21. Antibodies purified from the 30 mM sucrose supplemented culture had the highest levels of high mannose N-glycans, and lowest levels of fucosylated N-glycans. These antibodies also demonstrated the largest percent lysis of target cells, an increase of about 15 % compared to the cell lysis demonstrated with the unsupplemented control. Purified antibodies from the cultures supplemented with lower sucrose concentrations of 7 mM and 15 mM also facilitated an increase in percent lysis of target cells relative to the control, though the increase was lower when compared to the increase in percent lysis produced by antibodies purified from the cultures supplemented with 30 mM sucrose. These results indicate that not only can sucrose supplementation modulate the glycosylation profile of proteins, *e.g.*, antibodies, but the modulation has a beneficial impact on ensuring biologic comparability as well as therapeutic efficacy.

Discussion

Sucrose and tagatose are two sugars found in nature. Sucrose, in particular, has been found to be the most abundant sugar in soy beans (Hou A., *et al.*, Internat. J Agronomy (2009) Article ID 484571:8).

The data provided above demonstrate that the selective supplementation of sucrose and tagatose in host cell culture media is an effective approach for the targeted modulation of protein glycosylation profiles in cultured cell lines. In particular, supplementation of host cell culture media effectively modulated the N-glycan glycoform profile towards high mannose species, and decreased the overall level of fucosylated species. Since both sugars resulted in similar glycosylation profiles, it is likely that they modulate glycosylation via the same mechanism or pathway. Of the high mannose N-glycan species, Man 5 glycan increased the most upon exposure to either of the two sugars. Amongst the non-mannosylated N-glycan species, the largest increase was demonstrated in NGA2F-GlcNAc levels. The fact that both of Man 5 glycan and NGA2F-GlcNAc share the same UDP-GlcNAc co-substrate (Figure 2, highlighted) suggests that their accumulation may be due to inhibition of these reactions through a diminished supply of available UDP-GlcNAc substrate. The same shifting in protein glycosylation profiles was observed across multiple sucrose and tagatose concentrations in a concentration-dependent manner and across multiple cell lines expressing recombinant proteins. At select concentrations, an adverse impact of

these sugars on cell growth and productivity was minimized. The use of sucrose and/or tagatose in cell culture media provides an efficient and effective approach toward re-targeting of specific N-glycan glycoform profiles. This capability is important for ensuring biologic comparability, as well as the targeted optimization of product quality. The targeted increase in mannosylation levels, and decrease in fucosylation levels, is an important capability since for some recombinant glycoprotein therapeutics, high mannose glycans are a desired product characteristic. Furthermore, the decrease in overall fucosylation levels has been correlated with an increased ADCC response. High mannose glycans are also reported to be cleared faster from circulation (Alessandri, L., *et al.*, (2012) *mAbs Journal* **4**(4): 1-1210). The ability to modulate protein glycosylation through the selective use of monosaccharides and oligosaccharides (*e.g.*, sucrose and tagatose) is critically important for achieving critical product characteristics.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Patents, patent applications, publications, product descriptions, GenBank Accession Numbers, and protocols that may be cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties for all purposes. The contents of all cited references, including literature references, issued patents, and published patent applications, as cited throughout this application are hereby expressly incorporated herein by reference. It should further be understood that the contents of all the figures and tables attached hereto are expressly incorporated herein by reference. The entire contents of the following applications are also expressly incorporated herein by reference: U.S. Provisional Patent Application 61/893,123, entitled "STABLE SOLID PROTEIN COMPOSITIONS AND METHODS OF MAKING SAME", Attorney Docket Number 117813-31001, filed on October 18, 2013; U.S. Provisional Application Serial No. 61/892,833, entitled "LOW ACIDIC SPECIES COMPOSITIONS AND METHODS FOR PRODUCING THE SAME USING DISPLACEMENT CHROMATOGRAPHY", Attorney Docket Number 117813-

73602, filed on October 18, 2013; U.S. Provisional Patent Application 61/892,710, entitled
“MUTATED ANTI-TNF α ANTIBODIES AND METHODS OF THEIR USE”, Attorney
Docket Number 117813-73802, filed on October 18, 2013; U.S. Provisional Patent
Application 61/893,088, entitled “MODULATED LYSINE VARIANT SPECIES AND
5 METHODS FOR PRODUCING AND USING THE SAME”, Attorney Docket Number
117813-74101, filed on October 18, 2013; U.S. Provisional Patent Application 61/893,131,
entitled “PURIFICATION OF PROTEINS USING HYDROPHOBIC INTERACTION
CHROMATOGRAPHY”, Attorney Docket Number 117813-74301, filed on October 18,
2013; and U.S. Patent Application 14/077,871, entitled “LOW ACIDIC SPECIES
10 COMPOSITIONS AND METHODS FOR PRODUCING AND USING THE SAME”,
Attorney Docket Number 117813-73902, filed on November 12, 2013.

We claim:

1. A method of producing a composition comprising a protein with a modulated
5 glycosylation profile, said method comprising:
culturing a host cell expressing said protein in cell culture media supplemented with a monosaccharide and/or an oligosaccharide, thereby producing said composition comprising said protein with a modulated glycosylation profile as compared to a control,
wherein said control is a composition comprising a protein produced by culturing a
10 host cell expressing said protein in cell culture media which is not supplemented with a monosaccharide and/or an oligosaccharide.
2. The method of claim 1, further comprising purifying said composition comprising
15 said protein with a modulated glycosylation profile.
3. The method of claim 1, wherein the protein is an antibody or antigen-binding portion
thereof.
4. The method of claim 3, wherein the antibody is an anti-TNF α antibody.
20
5. The method of claim 4, wherein the anti-TNF α antibody is adalimumab, or antigen
binding fragment thereof.
6. The method of claim 1, wherein the protein is a dual variable domain immunoglobulin
25 (DVD-Ig).
7. The method of claim 1, wherein the protein is selected from the group consisting of a
TVD-IG, a half-body and a RAB.
- 30 8. The method of claim 1, wherein the monosaccharide is tagatose.
9. The method of claim 1, wherein the oligosaccharide is sucrose.
10. The method of claim 1, wherein the cell culture media is supplemented with a
35 sufficient amount of the monosaccharide to achieve a monosaccharide concentration selected

from the group consisting of about 1 mM, about 5 mM, about 7 mM, about 10 mM, about 20 mM, about 30 mM, about 40 mM, about 50 mM, about 60 mM, about 70 mM, about 80 mM, about 90 mM and about 100 mM.

5 11. The method of claim 10, wherein the monosaccharide concentration is 30 mM.

12. The method of claim 10 or 11, wherein the monosaccharide is tagatose.

10 13. The method of claim 1 wherein the cell culture media is supplemented with a sufficient amount of the oligosaccharide to achieve an oligosaccharide concentration selected from the group consisting of about 1 mM, about 5 mM, about 7 mM, about 10 mM, about 20 mM, about 30 mM, about 40 mM, about 50 mM, about 60 mM, about 70 mM, about 80 mM, about 90 mM and about 100 mM..

15 14. The method of claim 13, wherein the oligosaccharide concentration is 30 mM.

15. The method of claim 13 or 14, wherein the oligosaccharide is sucrose.

20 16. The method of claim 1, wherein the modulated glycosylation profile of the protein comprises modulation of a fucosylation level and/or a mannosylated N-glycan oligosaccharide level in said protein.

17. The method of claim 16, wherein the modulation of the fucosylation level comprises a decrease in the fucosylation level in said protein.

25

18. The method of claim 17, wherein the decrease in the fucosylation level comprises a decrease in the level of NGA2F, NA1F-GlcNAc, NA1F and/or NA2F in said protein.

30 19. The method of claim 18, wherein the decrease in the level of NGA2F, NA1F-GlcNAc, NA1F and/or NA2F is a decrease of about 0.1%, 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60% or 65%.

20. The method of claim 16, wherein the modulation of the fucosylation level comprises an increase in the fucosylation level in said protein.

21. The method of claim 20, wherein increase in the fucosylation level comprises an increase in the level of NGA2F-GlcNAc, NA1F-GlcNAc, NA1F and/or NA2F in said protein.

22. The method of claim 20, wherein the increase in the level of NGA2F-GlcNAc, NA1F-GlcNAc, NA1F or NA2F is an increase of about 0.1%, 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15% or 20%.

23. The method of claim 17, wherein the overall decrease in the fucosylation level comprises an increase or a decrease in the level of NGA2F, NGA2F-GlcNAc, NA1F-GlcNAc, NA1F and/or NA2F in said protein.

24. The method of claim 23, wherein in the overall decrease in the fucosylation level is a decrease of about 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45% or 50%.

25. The method of claim 16, wherein the modulation of the mannosylated N-glycan level comprises an increase in the mannosylation level of said protein.

26. The method of claim 25, wherein the increase in the mannosylation level is an increase of about 0.1%, 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45% or 50%.

27. The method of claim 25, wherein the increase in the mannosylation level comprises an increase in the level of a high mannose N-glycan oligosaccharide selected from the group consisting of Man 5 glycan, Man 6 glycan, Man 7 glycan and Man 8 glycan.

28. The method of claim 27, wherein the levels of Man 5 glycan, Man 6 glycan, Man 7 glycan and/or Man 8 glycan are increased by about 0.1%, 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40, 45% or 50%.

29. The method of claim 1, wherein said host cell is a CHO cell.

30. A method of producing a composition comprising an antibody, or antigen binding fragment thereof, with a modulated glycosylation profile, said method comprising:

5 culturing a host cell expressing said antibody, or antigen binding fragment thereof, in cell culture media supplemented with sucrose and/or tagatose, thereby producing said composition comprising said antibody, or antigen binding fragment thereof, with an increased level of mannosylated N-glycans and a decreased level of fucosylated N-glycans as compared to a control,

10 wherein said control is a composition comprising an antibody, or antigen binding fragment thereof, produced by culturing a host cell expressing said antibody, or antigen binding fragment thereof, in cell culture media which is not supplemented with tagatose and/or glucose.

15 31. The method of claim 30, wherein the antibody is adalimumab, or antigen binding fragment thereof.

32. A method of producing a composition comprising an antibody, or antigen binding fragment thereof, with a modulated glycosylation profile, said method comprising:

20 culturing a host cell expressing said antibody, or antigen binding fragment thereof, in cell culture media supplemented with sucrose and/or tagatose, thereby producing said composition comprising said antibody, or antigen binding fragment thereof, with a 1-50% increase in the level of mannosylated N-glycans and a 1-50% decrease in the level of fucosylated N-glycans as compared to a control,

25 wherein said control is a composition comprising an antibody, or antigen binding fragment thereof, produced by culturing a host cell expressing said antibody, or antigen binding fragment thereof, in cell culture media which is not supplemented with tagatose and/or glucose.

30 33. The method of claim 32, wherein the antibody is adalimumab, or antigen binding fragment thereof.

34. A composition comprising a cell culture media comprising a monosaccharide and/or an oligosaccharide.

35. The composition of claim 34, wherein the monosaccharide is tagatose.

36. The composition of claim 34, wherein the oligosaccharide is sucrose.

5

37. A pharmaceutical composition comprising the composition produced by the methods of any one of claims 1, 30 or 32 and a pharmaceutically acceptable carrier.

38. A composition comprising a therapeutic protein with a modulated glycosylation profile produced by the methods of any one of claims 1, 30 or 32.

10

39. The composition of claim 38, wherein the therapeutic protein is an antibody.

40. A composition comprising a therapeutic protein, wherein said protein comprises a 1-50% increase in the level of mannosylated N-glycans and a 1-50% decrease in the level of fucosylated N-glycans, as compared to a control,

15

wherein said control is a composition comprising a protein produced by culturing a host cell expressing said protein in cell culture media which is not supplemented with a monosaccharide and/or an oligosaccharide

20

41. The composition of claim 40, wherein the therapeutic protein is selected from the group consisting of an antibody, an antigen-binding portion thereof, DVD-Ig, TVD-Ig, RAB and half-body.

25 42. A composition of claim 40, wherein the therapeutic protein is an antibody.

43. A method of producing a composition comprising an antibody, or antigen binding fragment thereof, with a modulated glycosylation profile, said method comprising:

culturing a host cell expressing said antibody, or antigen binding fragment thereof, in cell culture media supplemented with sucrose and/or tagatose, thereby producing said composition comprising said antibody, or antigen binding fragment thereof, with a 1-30% increase in antibody-dependent cellular cytotoxicity (ADCC) response as compared to a control,

30

wherein said control is a composition comprising an antibody, or antigen binding fragment thereof, produced by culturing a host cell expressing said antibody, or antigen binding fragment thereof, in cell culture media which is not supplemented with tagatose and/or glucose.

5

44. The method of claim 43, wherein the antibody is adalimumab, or antigen binding fragment thereof.

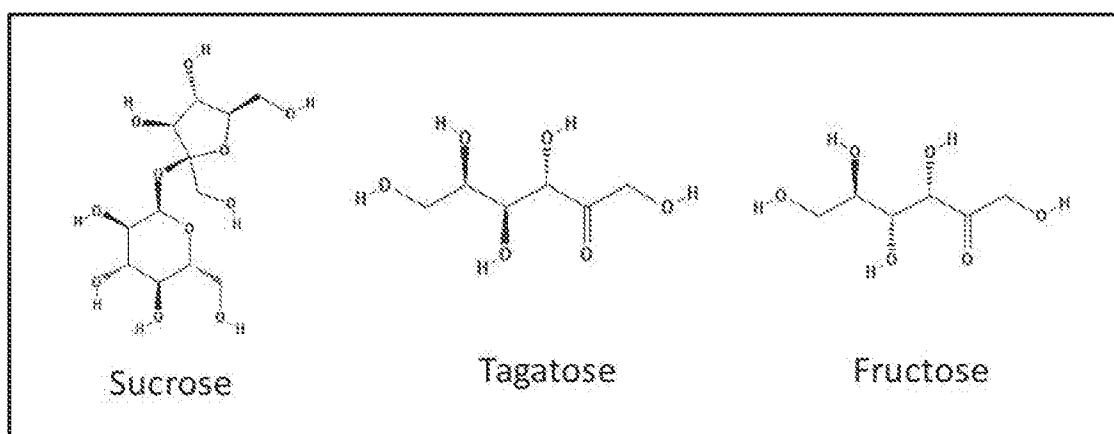


Figure 1

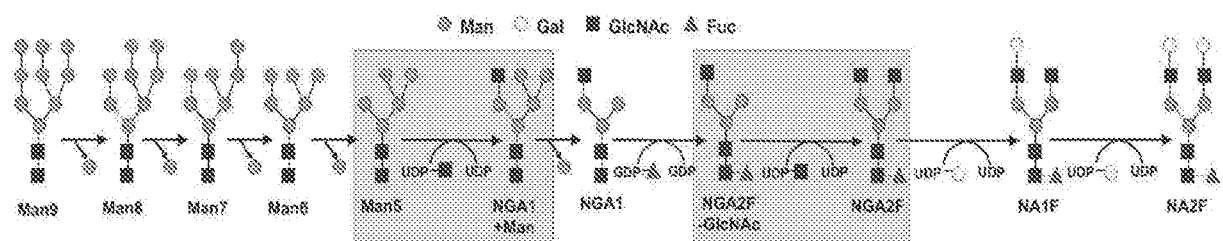


Figure 2

Figure 3A

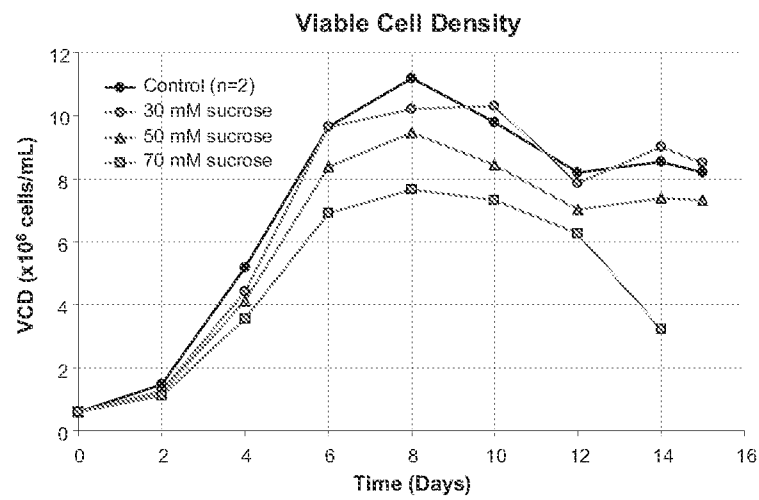


Figure 3B

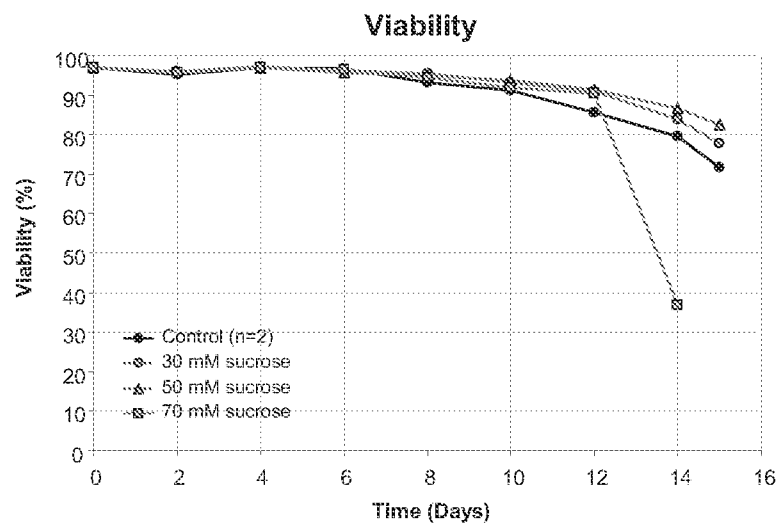
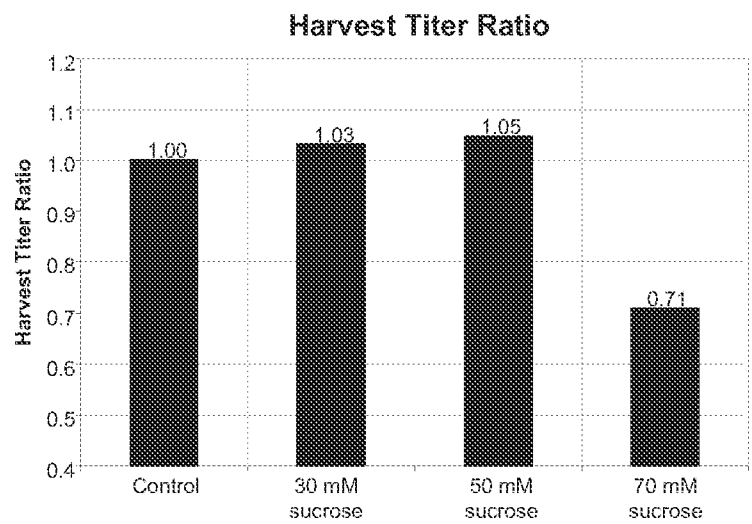


Figure 3C



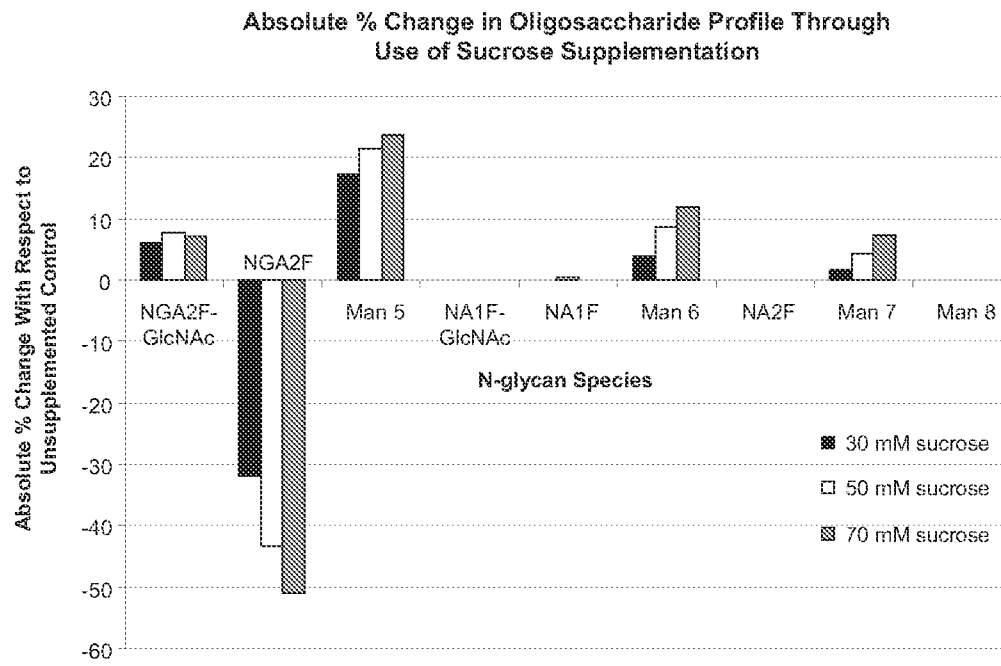


Figure 4

Figure 5A

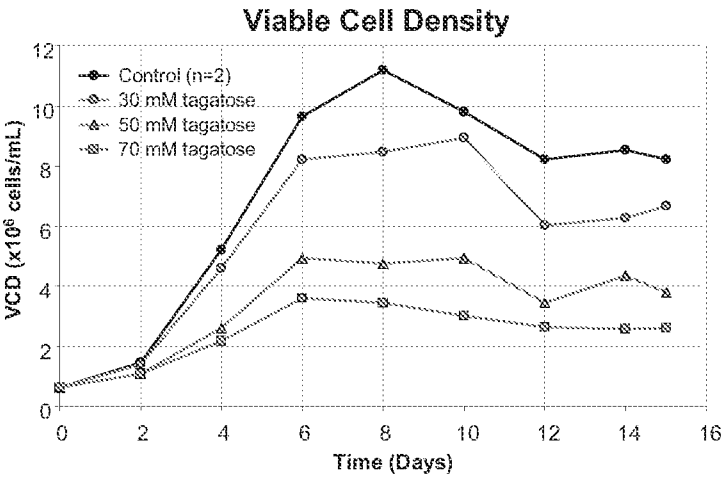


Figure 5B

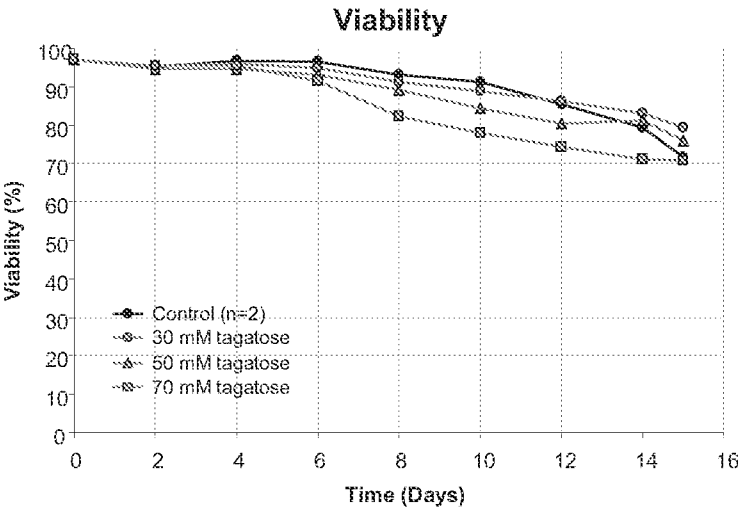
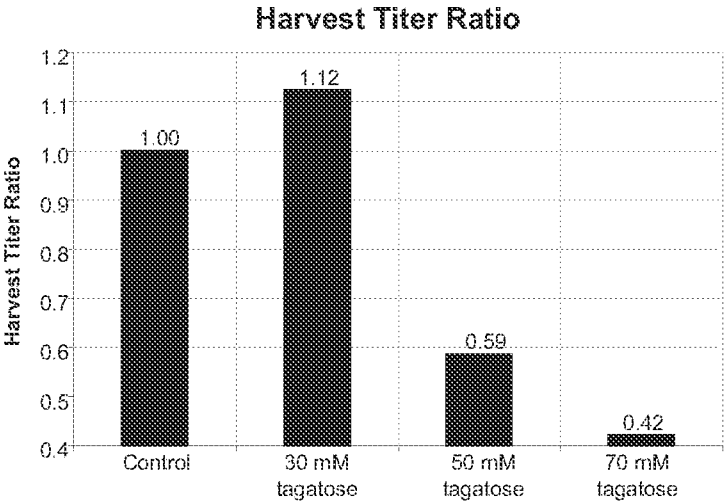


Figure 5C



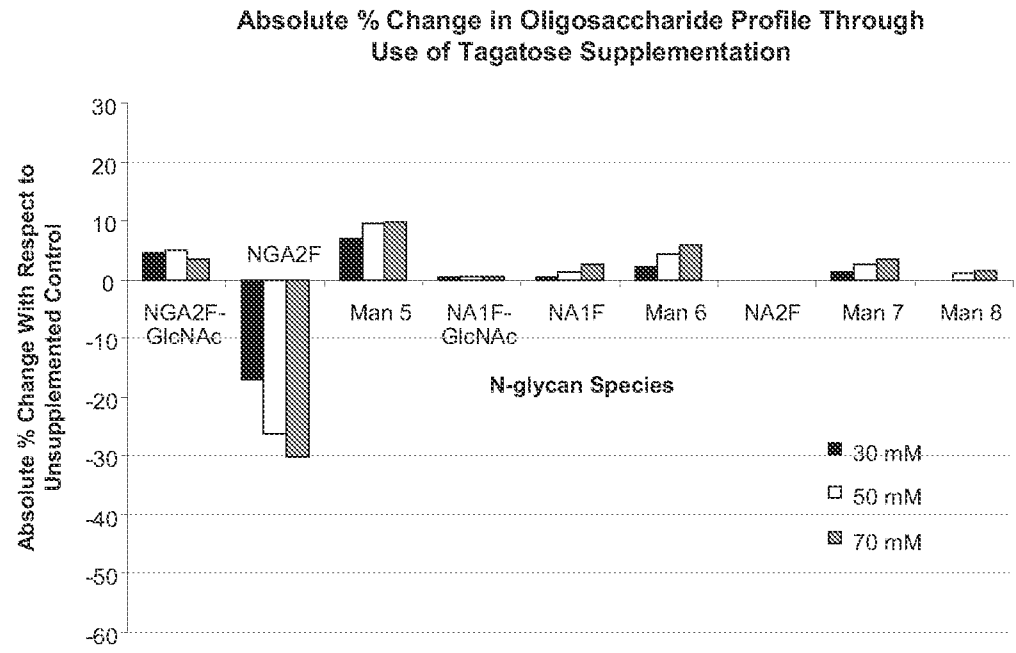


Figure 6

Figure 7A

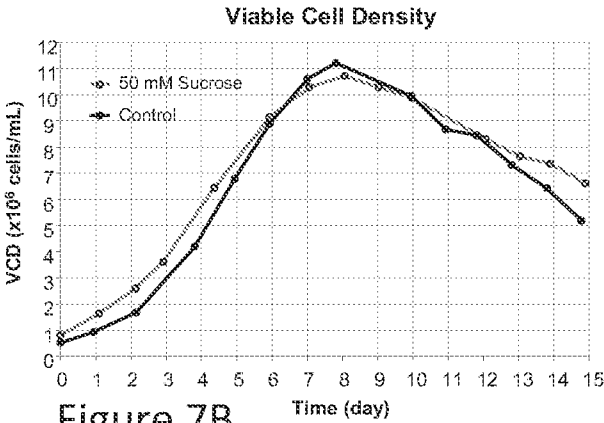


Figure 7D

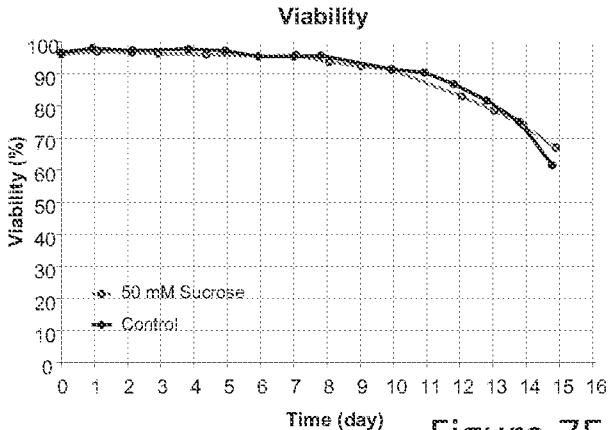


Figure 7B

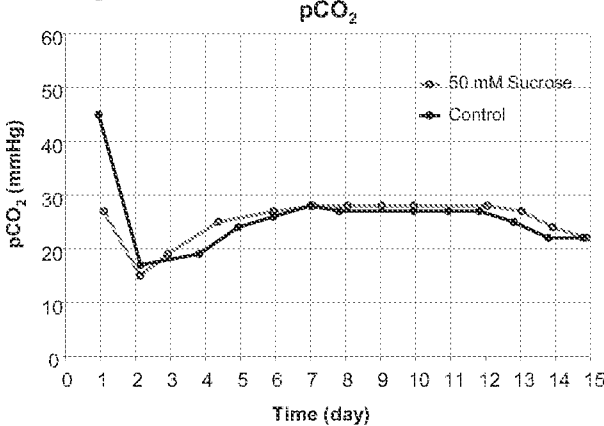


Figure 7E

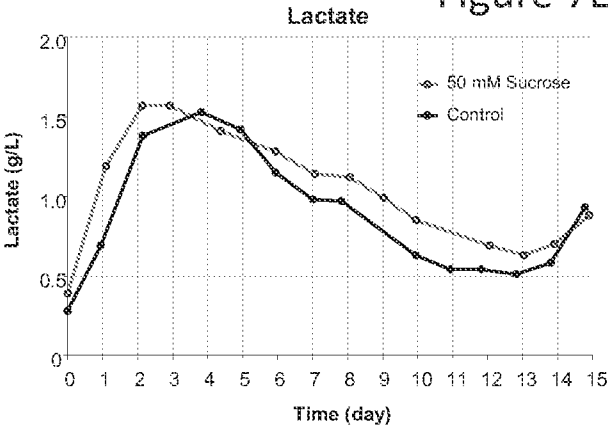


Figure 7C

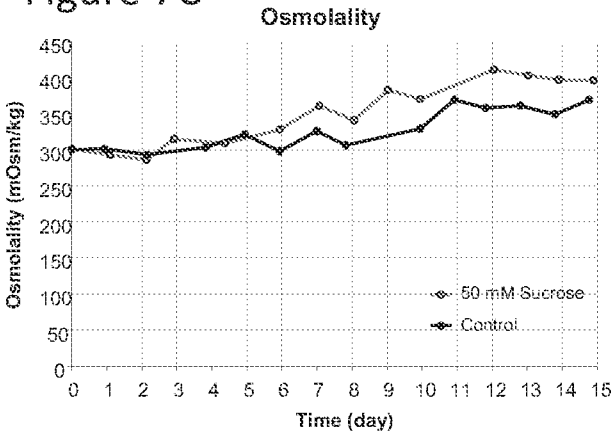
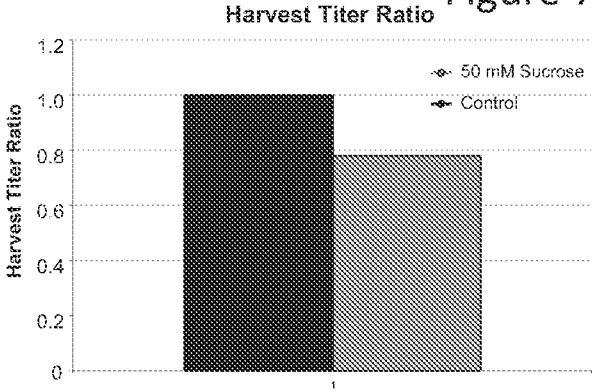


Figure 7F



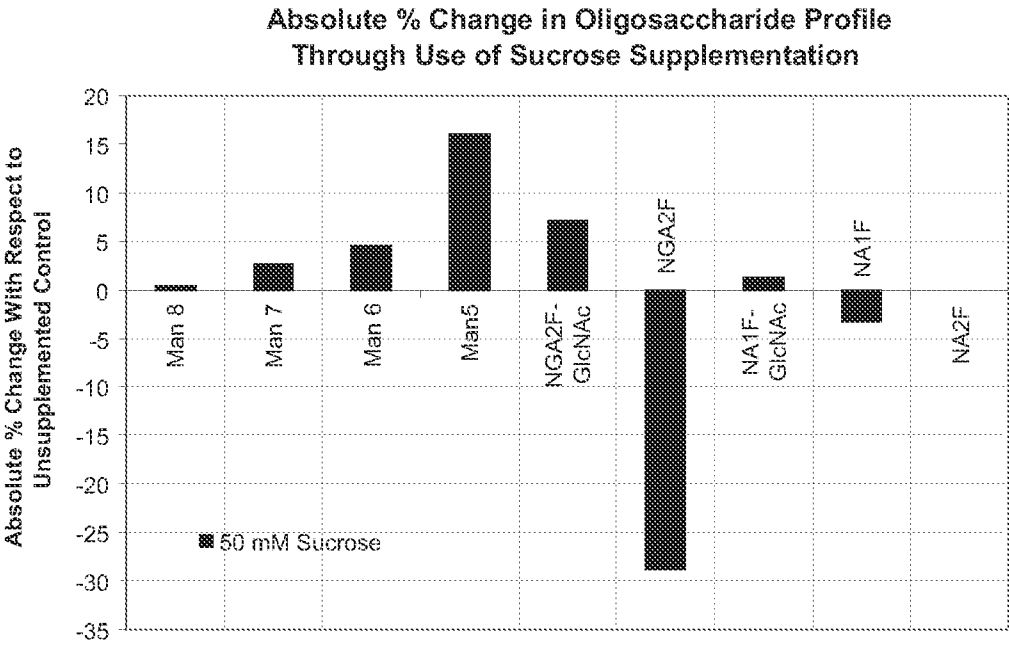


Figure 8

Figure 9A

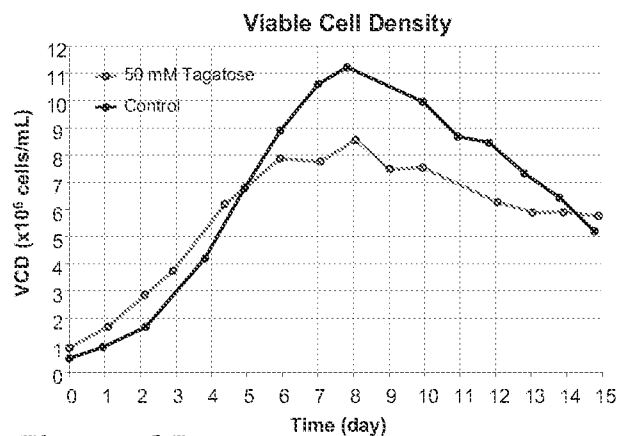


Figure 9D

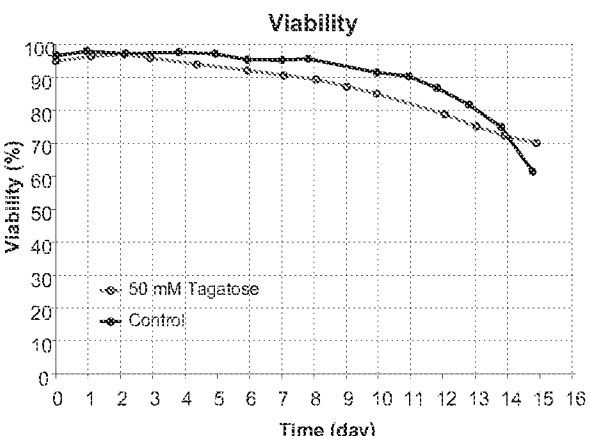


Figure 9B

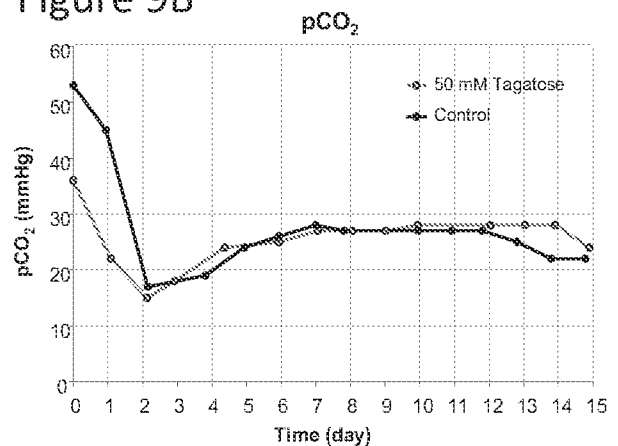


Figure 9E

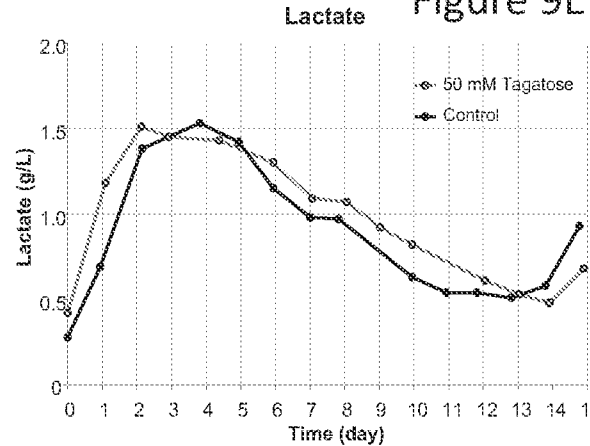


Figure 9C

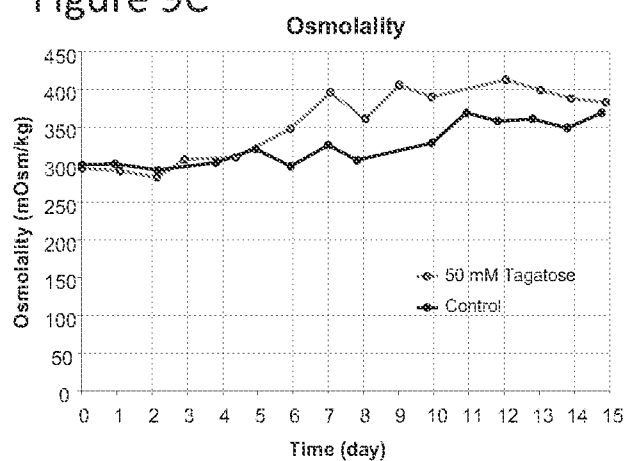
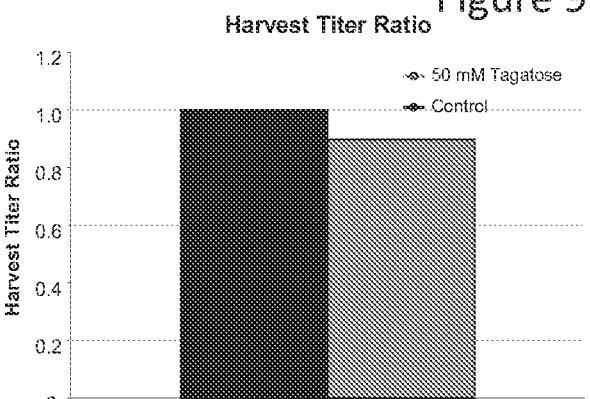


Figure 9F



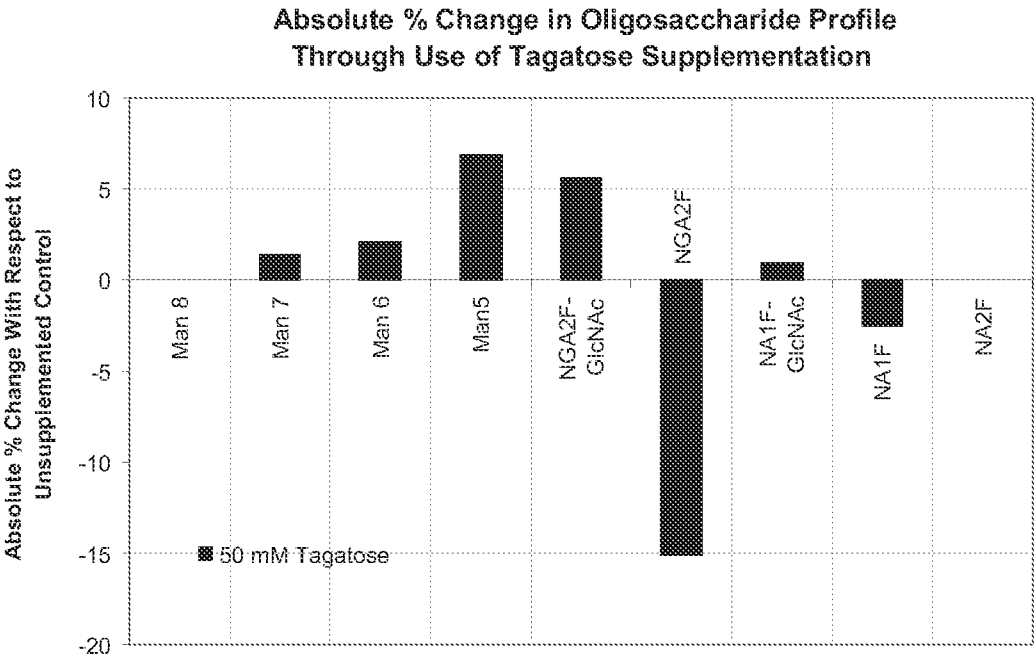


Figure 10

Figure 11A

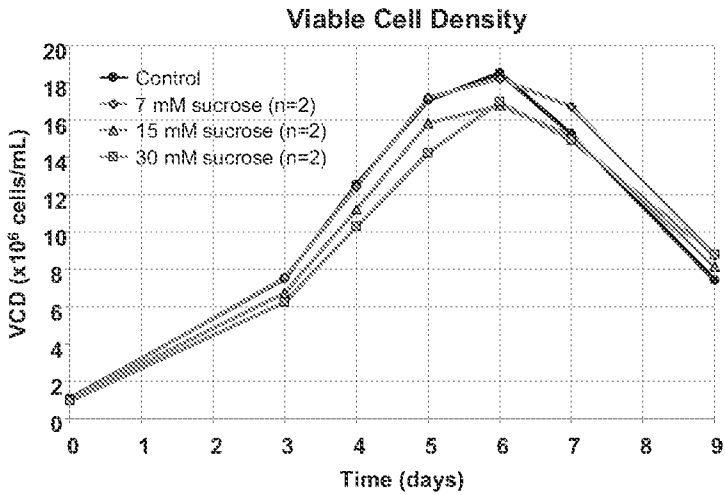


Figure 11B

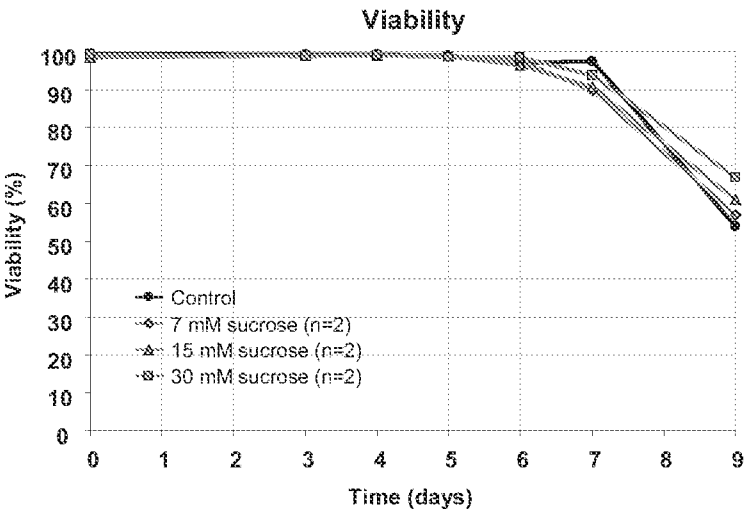
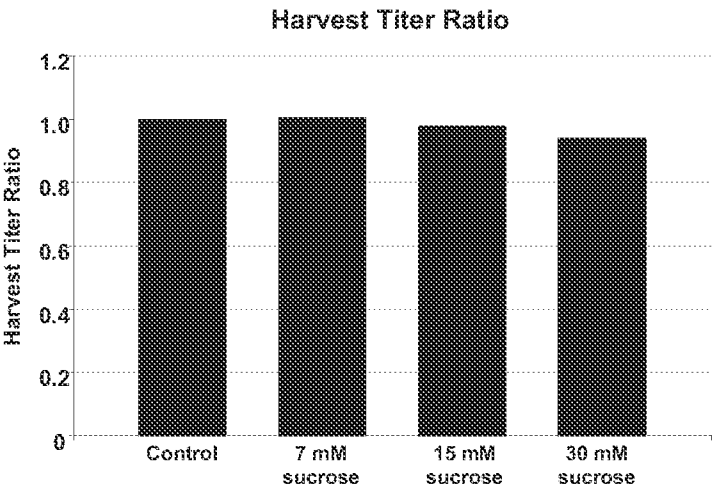


Figure 11C



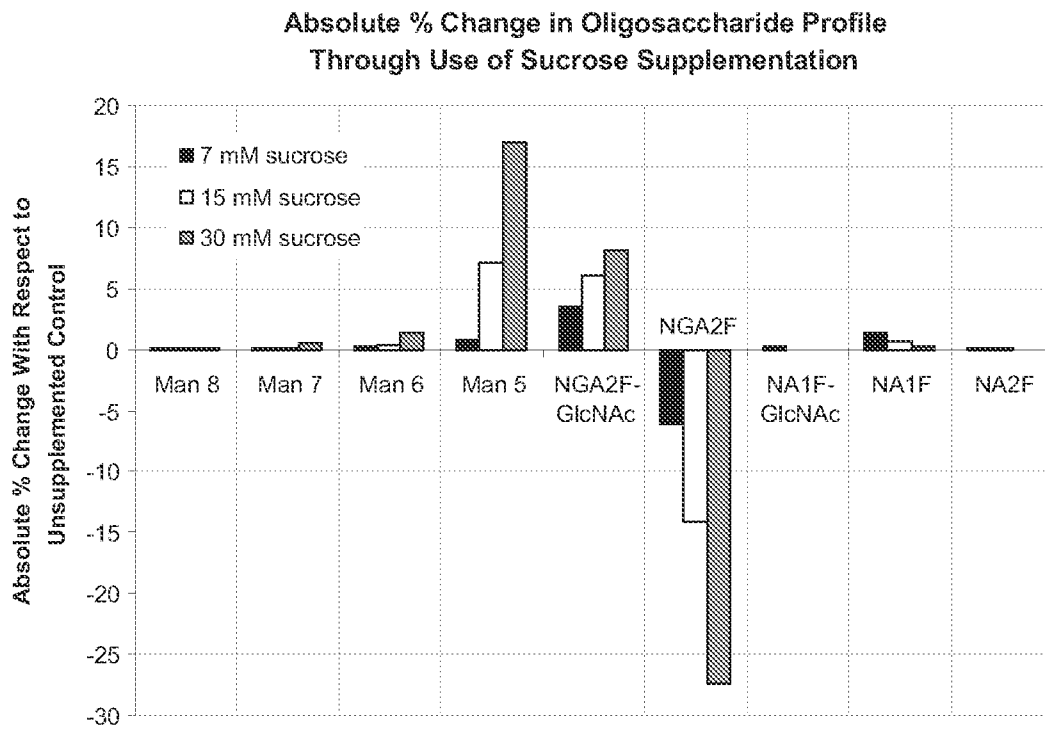


Figure 12

Fig. 13B

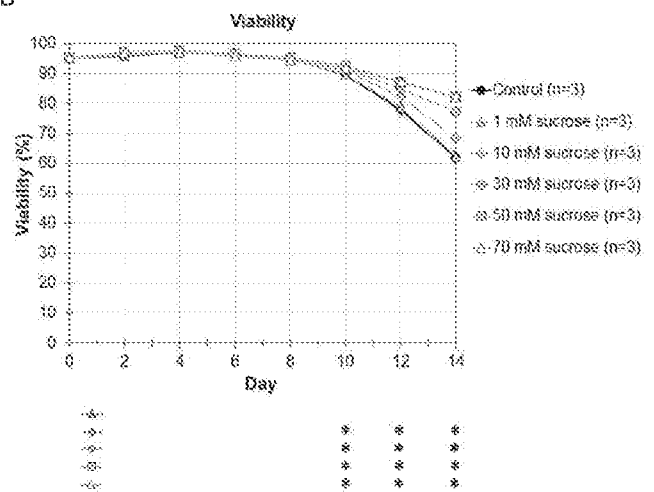


Fig. 13D

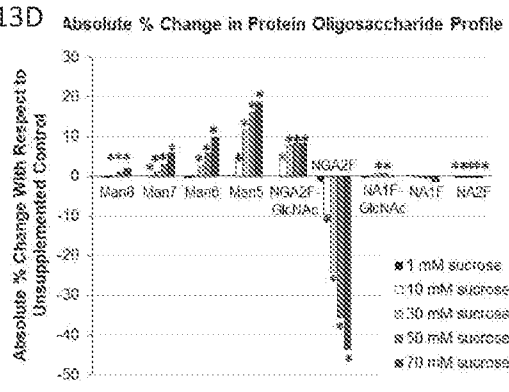


Fig. 14A

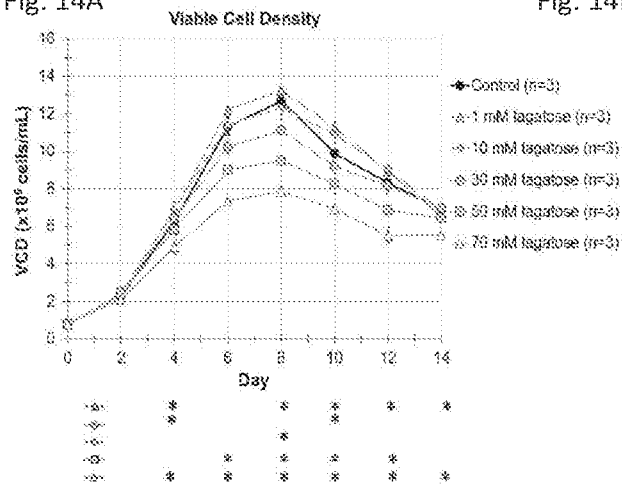


Fig. 14B

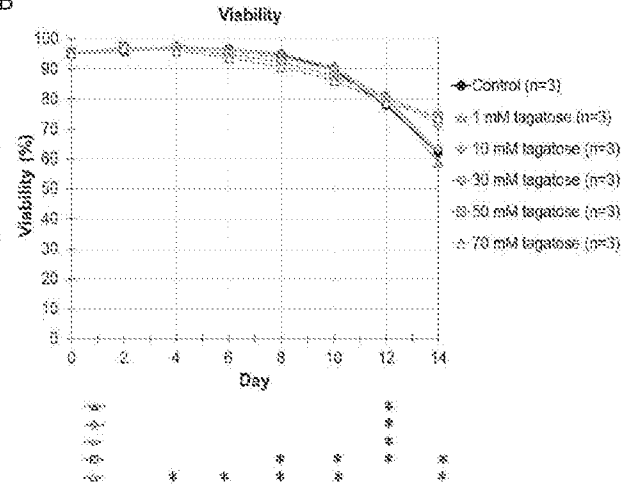


Fig. 14C

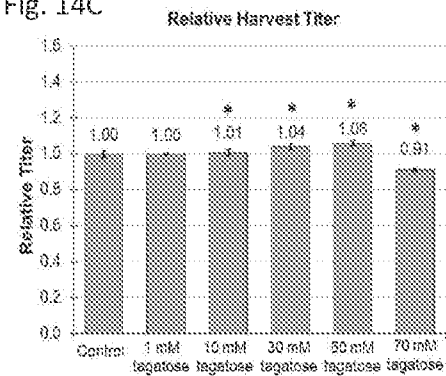


Fig. 14D

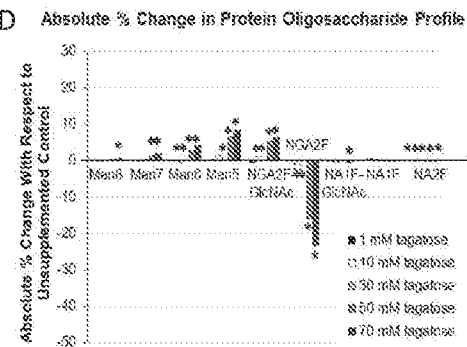


Fig. 15A

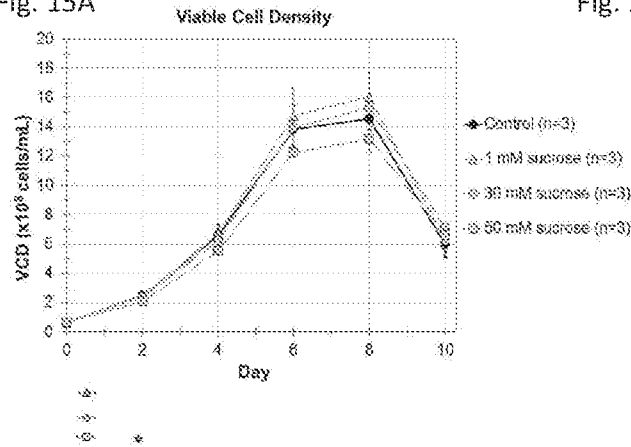


Fig. 15B

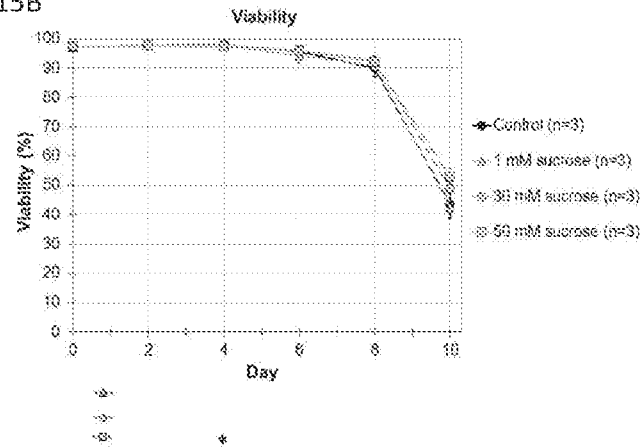


Fig. 15C

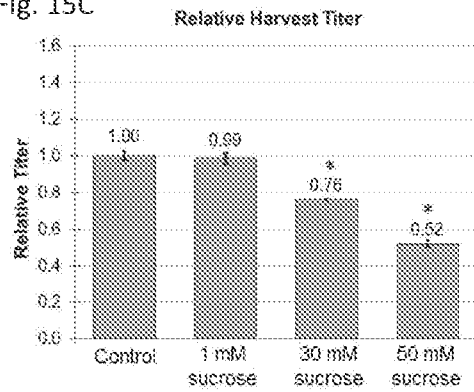


Fig. 15D

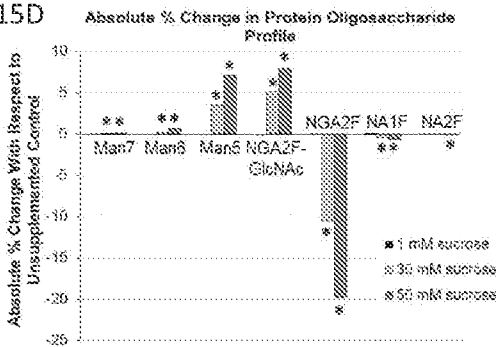


Fig. 16A

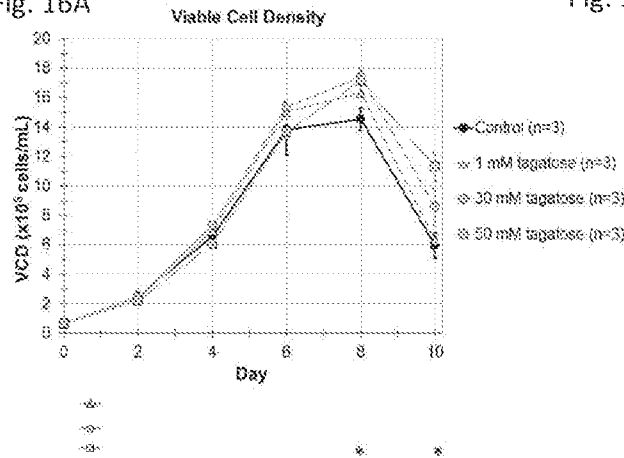


Fig. 16B

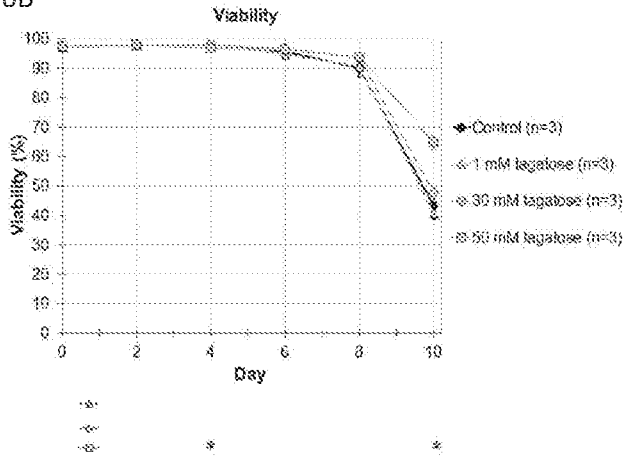


Fig. 16C

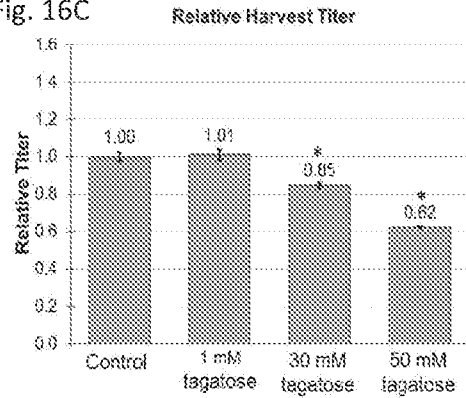


Fig. 16D

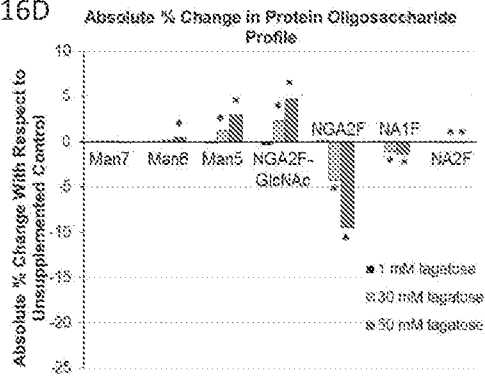


Fig. 17A

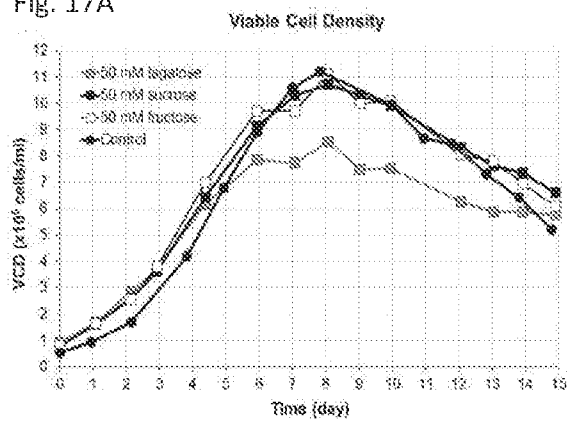


Fig. 17B

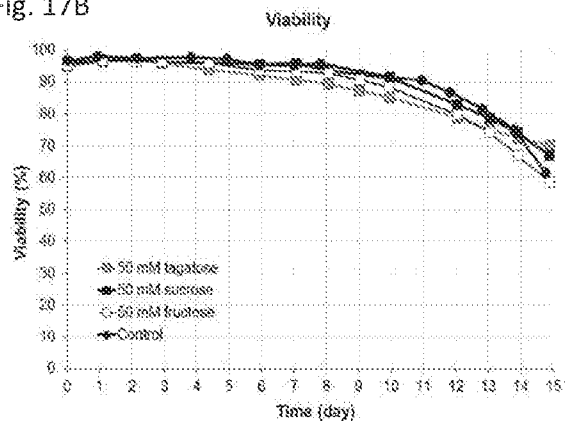


Fig. 17C

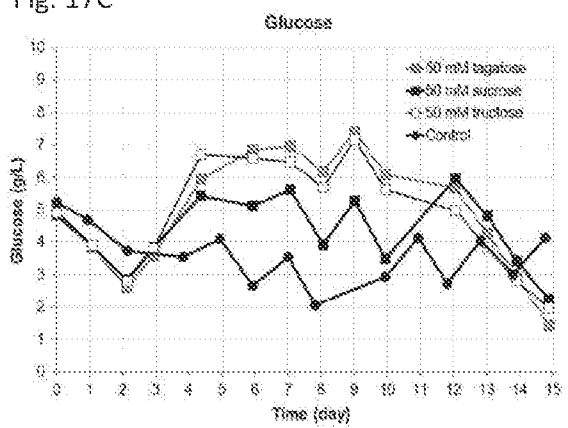


Fig. 17D

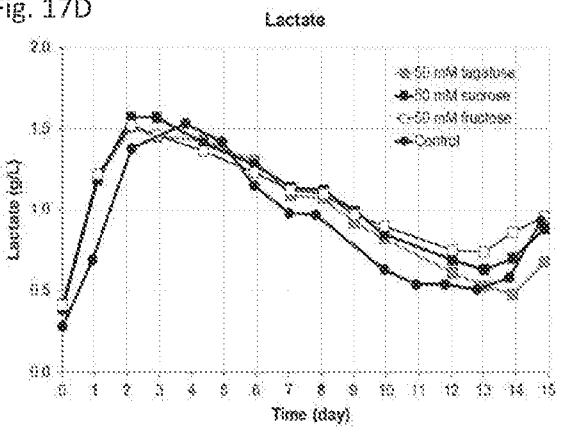


Fig. 17E

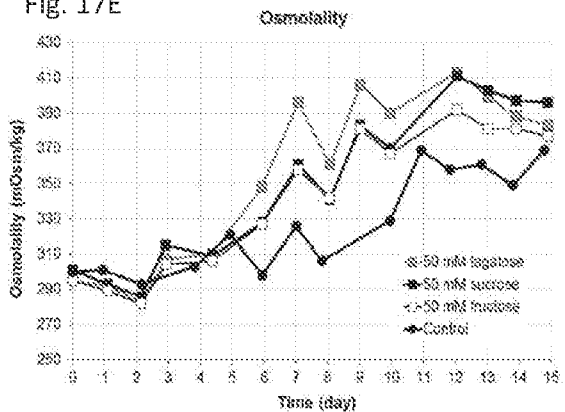
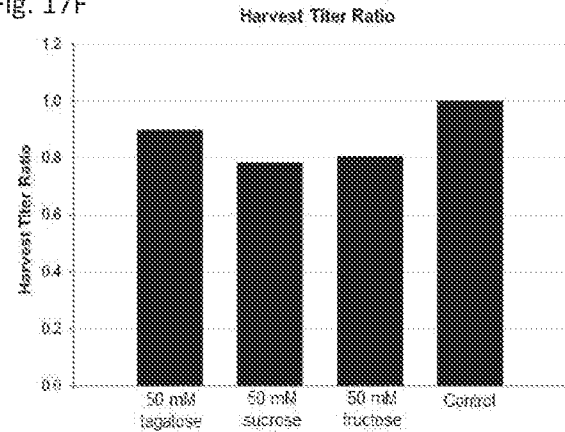


Fig. 17F



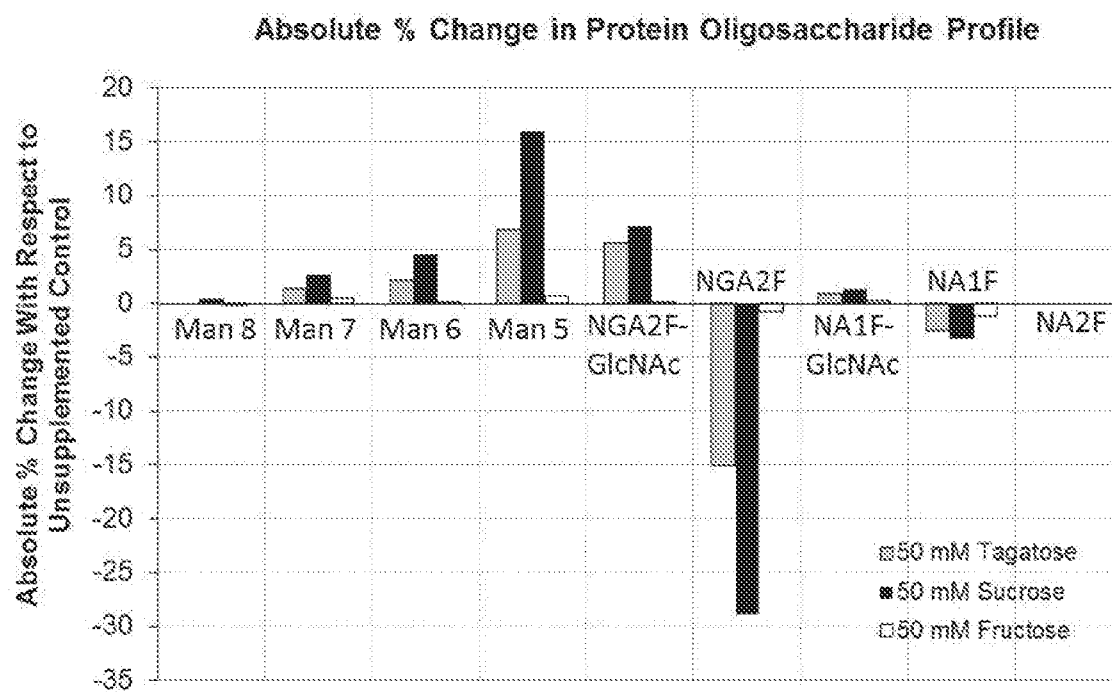


Figure 18

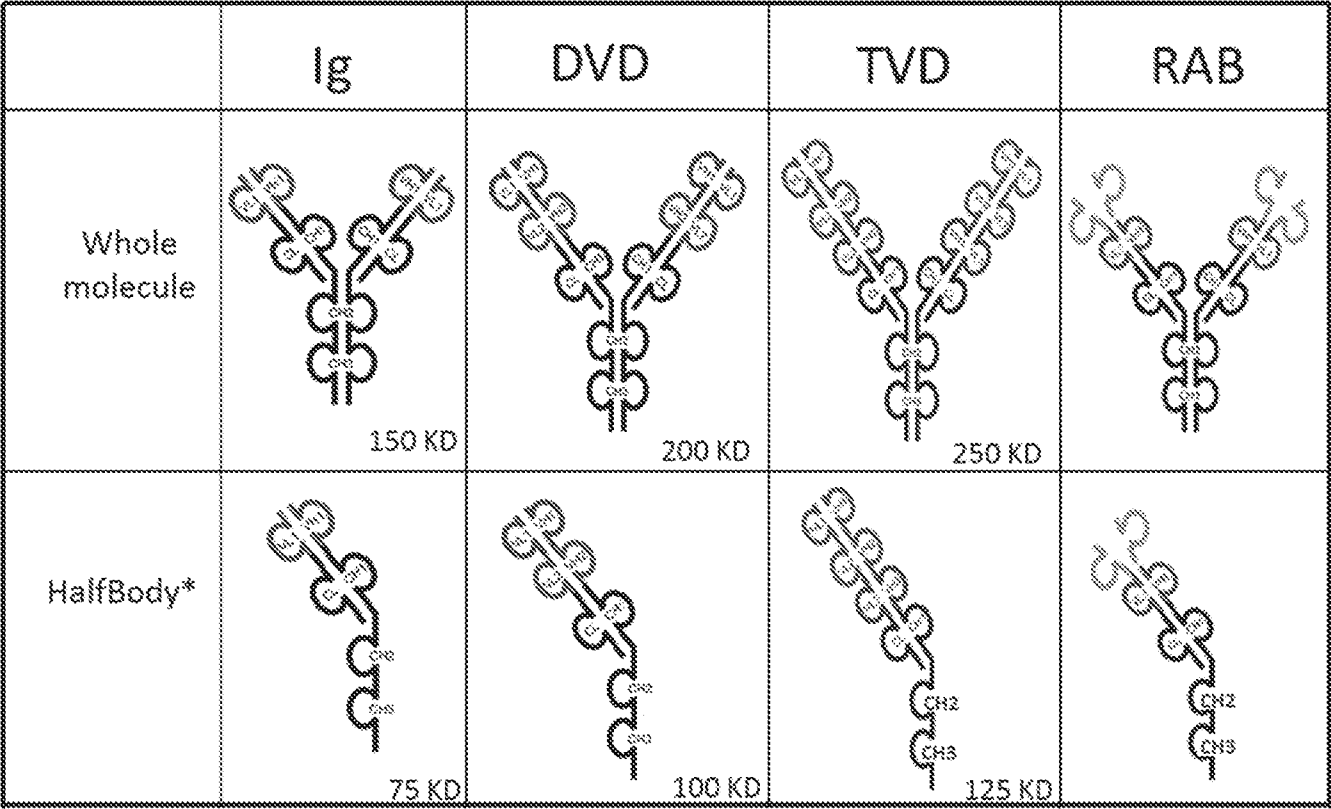


Figure 19

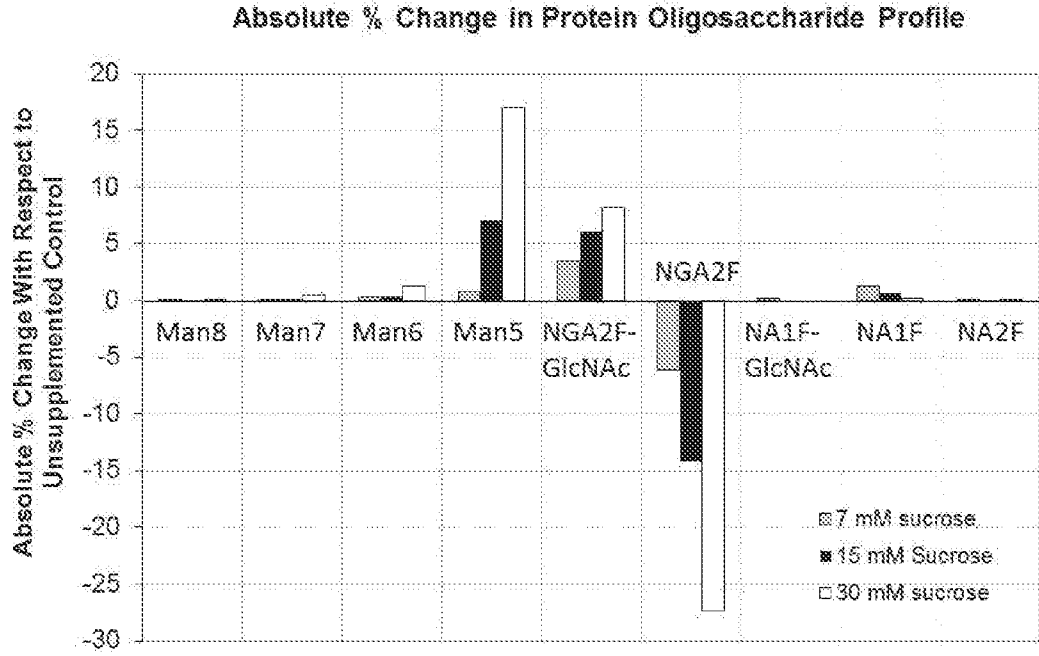


Figure 20

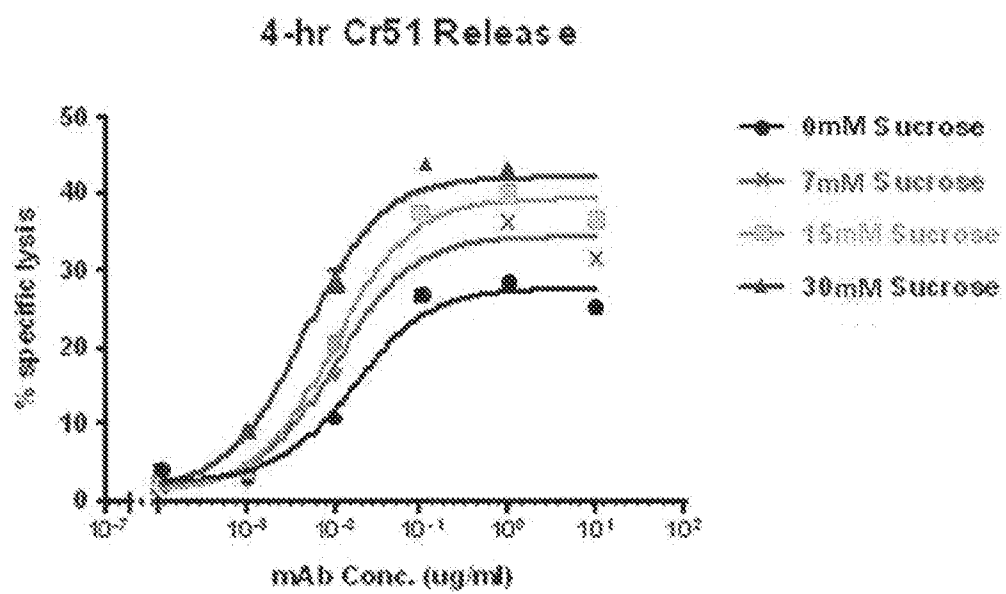


Figure 21