(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 October 2016 (13.10.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/162514 A1

(51) International Patent Classification:
C07K 16/00 (2006.01)

(21) International Application Number:
PCT/EP2016/057818

(22) International Filing Date:
8 April 2016 (08.04.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
15163200.7 10 April 2015 (10.04.2015) EP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*

WO 2016/162514 A1

(54) Title: METHODS FOR MODULATING PROTEIN GLYCOSYLATION PROFILES OF RECOMBINANT PROTEINS

(57) Abstract: The present invention relates to methods and compositions for modulating glycosylation profile of recombinant proteins expressed by mammalian host cells during the cell culture process by supplementing cell culture media with a trisaccaride.

METHODS FOR MODULATING PROTEIN GLYCOSYLATION PROFILES OF RECOMBINANT PROTEINS

FIELD OF THE INVENTION

The present invention relates to methods and compositions for modulating glycosylation profile of recombinant proteins expressed by mammalian host cells during the cell culture process.

BACKGROUND OF THE INVENTION

The glycosylation profile of a protein, such as a therapeutic protein or an antibody, is an important characteristic that influences biological activity of the protein through changes in half-life and affinity due to effects for instance on folding, stability and antibody-dependent cellular cytotoxicity (ADCC, one of the mechanism responsible for the therapeutic effect of antibodies). Glycosylation is highly dependent on the cell line that is used for the production of the protein of interest, as well as on the cell culture processes (pH, temperature, cell culture media composition, raw material lot-to-lot variation, medium filtration material, air, etc).

ADCC activity is influenced by the amount of fucose and/or mannose linked to the oligosaccharides of the Fc region, with enhanced activity seen with a reduction in fucose and/or an increase in mannose. Indeed, for instance, compared to fucosylated IgGs, non-fucosylated forms exhibit dramatically enhanced ADCC due to the enhancement of FcγRIIIa binding capacity without any detectable change in complement-dependent cytotoxicity (CDC) or antigen binding capability (Yamane-Ohnuki and Satoh, 2009). Similarly, antibodies exhibiting high level of mannose-5 glycans also presented higher ADCC (Yu et al., 2012). Thus, where the ADCC response is the principle therapeutic mechanism of antibody activity, the provision of methods for the preparation of recombinant therapeutic protein with a glycosylation profile characterized by decreased fucosylation and/or increased mannosylation, are beneficial. The advantages of non-fucosylated and/or highly mannosylated antibodies also include achieving therapeutic efficacy at low doses. However, many therapeutic antibodies that are currently on the market are heavily fucosylated because they are produced by mammalian cell lines with intrinsic enzyme activity responsible for the core-fucosylation of the Fc N-glycans of the products.

Modulation of protein glycosylation is of particular relevance for marketed therapeutic proteins or antibodies as glycosylation (such as mannosylation and/or fucosylation) can impact therapeutic utility and safety. Further, in the frame of biosimilar compounds, control of the glycosylation profile of a recombinant protein is crucial, as the glycosylation profile of said recombinant protein has to be comparable to the glycosylation profile of the reference product.

Therefore, there remains a need for culture conditions and production methods that allow controling the glycosylation profile, such as fucosylation and/or mannosylation profiles, of a recombinant protein. The present invention addresses this need by providing methods and compositions for modulating recombinant protein glycosylation.

SUMMARY OF THE INVENTION

In one aspect the invention provides a method of producing a recombinant protein with a modulated glycosylation profile, said method comprising culturing a host cell expressing said protein in cell culture medium supplemented with a trisaccharide.

Alternatively, here is disclosed a method of producing a recombinant protein with a modulated glycosylation profile, said method comprising culturing a host cell expressing said protein in cell culture medium complemented with at least one feed comprising a trisaccharide

In a further aspect, the invention provides a composition comprising a cell culture medium comprising a trisaccharide.

In another aspect, the invention provides a pharmaceutical composition comprising the recombinant protein with a modulated glycosylation profile produced by the methods of the invention and a pharmaceutically acceptable carrier.

In another aspect, the invention provides a composition comprising a recombinant protein with a modulated glycosylation profile produced by the methods of the invention.

In a further aspect, the invention provides use of a trisaccharide for modulating the glycosylation profile of recombinant proteins.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows density of viable cells (Figure 1A; Guava®) and viability (Figure 1B; Guava®) in relation to time and titer (Figure 1C; Octet®) on day 14 for mAb1 cells cultured at different raffinose concentrations in microplates. Results are presented as mean±standard deviation. Figure 1D is the legend. The concentrations which are mentioned refer to the concentrations at day 0, just after the inoculation.

Figure 2 shows analysis of quality of mAb1 antibody secreted at different raffinose concentrations in microplates. Fucosylation and glycosylation profiles obtained by CGE-LIF analysis are presented at left (respectively Figure 2A and Figure 2C), whereas the change comparing to the control is shown on the right (respectively Figure 2B and Figure 2D). The legend is identical for any one of figures 2A to 2D. In any one of figures 2A to 2D, for each type of glycans the results are presented from the lowest dose to the highest dose of raffinose.

Figure 3 shows density of viable cells (Figure 3A; Guava®) and viability (Figure 3A; Guava®) in relation to time and titer (Figure 3C; Octet®) on day 14 for mAb2 cells cultured at different concentrations of raffinose in microplates. Results are presented as mean±standard deviation. Figure 3D is the legend.

Figure 4 shows analysis of quality of mAb2 antibody secreted at different raffinose concentrations in microplates. Fucosylation and glycosylation profiles obtained by CGE-LIF analysis are presented at left (respectively Figure 4A and Figure 4C), whereas the change comparing to the control is shown on the right (respectively Figure 4B and Figure 4D). The legend is identical for any one of figures 4A to 4D. In any one of figures 4A to 4D, for each type of glycans the results are presented from the lowest dose to the highest dose of raffinose.

DETAILED DESCRIPTION OF THE INVENTION

All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. The publications and applications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in art to which the subject matter herein belongs. As used herein, the following definitions are supplied in order to facilitate the understanding of the present invention.

As used in the specification and claims, the term "and/or" used in a phrase such as "A and/or B" herein is intended to include "A and B", "A or B", "A", and "B".

As used in the specification and claims, the term "cell culture" or "culture" is meant the growth and propagation of cells in vitro, i.e. outside of an organism or tissue. Suitable culture conditions for mammalian cells are known in the art, such as taught in Cell Culture Technology for Pharmaceutical and Cell-Based Therapies (2005). Mammalian cells may be cultured in suspension or while attached to a solid substrate. .

The terms “cell culture medium,” “culture medium”, “medium,” and any plural thereof, refer to any medium in which cells of any type can be cultured. A “basal medium” refers to a cell culture medium that contains all of the essential ingredients useful for cell metabolism. This includes for instance amino acids, lipids, carbon source, vitamins and mineral salts. DMEM (Dulbeccos’ Modified Eagles Medium), RPMI (Roswell Park Memorial Institute Medium) or medium F12 (Ham’s F12 medium) are examples of commercially available basal media. Alternatively, said basal medium can be a proprietary medium fully developed in-house, also herein called “chemically defined medium” or “chemically defined culture medium”, in which all of the components can be described in terms of the chemical formulas and are present in known concentrations. The culture medium can be free of proteins and/or free of serum, and can be supplemented by any additional compound(s) such as amino acids, salts, sugars, vitamins, hormones, growth factors, depending on the needs of the cells in culture.

The term “feed medium” (and plural thereof) refers to a medium used as a supplementation during culture to replenish the nutrients which are consumed. The feed medium can be a commercially available feed medium or a proprietary feed medium (herein alternatively chemically defined feed medium).

The term “bioreactor” or “culture system” refers to any system in which cells can be cultured, preferably in batch or fed-batch mode. This term includes but is not limited to flasks, static flasks, spinner flasks, tubes, shake tubes, shake bottles, wave bags, bioreactors, fiber bioreactors, fluidized bed bioreactors, and stirred-tank bioreactors with or without microcarriers. Alternatively, the term “culture system” also includes microtiter plates, capillaries or multi-well plates. Any size of bioreactor can be used, for instance from 0.1 milliliter (0.1 mL, very small scale) to 20000 liters (20000L or 20

KL, large scale), such as 0.1 mL, 0.5 mL 1 mL, 5 mL, 0.01L, 0.1L, 1L, 2L, 5L, 10L, 50L, 100L, 500L, 1000L (or 1KL), 2000L (or 2K), 5000L (or 5KL), 10000L (or 10KL), 15000L (or 15KL) or 20000L (20KL).

The term “fed-batch culture” refers to a method of growing cells, where there is a bolus or continuous feed media supplementation to replenish the nutrients which are consumed. This cell culture technique has the potential to obtain high cell densities in the order of greater than 10 x 10^6 to 30 x 10^6 cells/ml, depending on the media formulation, cell line, and other cell growth conditions. A biphasic culture condition can be created and sustained by a variety of feed strategies and media formulations.

Alternatively a perfusion culture can be used. Perfusion culture is one in which the cell culture receives fresh perfusion feed medium while simultaneously removing spent medium. Perfusion can be continuous, step-wise, intermittent, or a combination of any or all of any of these. Perfusion rates can be less than a working volume to many working volumes per day. Preferably the cells are retained in the culture and the spent medium that is removed is substantially free of cells or has significantly fewer cells than the culture. Perfusion can be accomplished by a number of cell retention techniques including centrifugation, sedimentation, or filtration (see for example Voisard et al., 2003).

When using the methods and/or cell culture techniques of the instant invention, the protein with a modulated glycosylation profile are generally directly secreted into the culture medium. Once said protein is secreted into the medium, supernatants from such expression systems can be first concentrated using a commercially available protein concentration filter.

As used herein, "cell density" refers to the number of cells in a given volume of culture medium. "Viable cell density" refers to the number of live cells in a given volume of culture medium, as determined by standard viability assays. The cell density will be considered as maintained if it is in the range of -15% to 15% compared to the control culture condition.

The term “viability”, or “cell viability” refers to the ratio between the total number of viable cells and the total number of cells in culture. Viability is usually acceptable as long as it is at not less than 60 % compared to the start of the culture (however, the acceptable threshold can be determined case by case). Viability is often used to determine time for harvest. For instance, in fed-batch culture, harvest can be performed once viability reaches at 60% or after 14 days in culture.

The wording “titre” refers to the amount or concentration of a substance, here the protein of interest, in solution. It is an indication of the number of times the solution can be diluted and still contain detectable amounts of the molecule of interest. It is calculated routinely for instance by diluting serially (1:2, 1:4, 1:8, 1:16, etc) the sample containing the protein of interest and then using appropriate detection method (colorimetric, chromatographic etc.), each dilution is assayed for the presence of detectable levels of the protein of interest. Titre can also be measured by means such as by fortéBIO Octet® or with Biacore C®, as used in the example section. The term “specific productivity” refers to the amount of a substance, here the protein of interest, produced per cell per day. The titre or specific productivity will be considered as maintained if it is in the range of -10% to 10% compared to the control culture condition.

As used in the specification and claims, a "modulated glycosylation profile" includes a glycosylation profile of a recombinant protein (for example a therapeutic protein or antibody) that is modulated as

compared to the glycosylation profile of that same protein produced by culturing a recombinant cell expressing that recombinant protein in cell culture media which is not supplemented with a trisaccharide, such as raffinose. The modulated glycosylation profile may include modulation of a fucosylation level and/or a mannosylation level in said protein. In an embodiment, the modulated glycosylation profile may include an overall increase in the level of mannosylation and an overall decrease in the level of fucosylation of the protein.

The term “protein” as used herein includes peptides and polypeptides and refers to compound comprising two or more amino acid residues. A protein according to the present invention includes but is not limited to a cytokine, a growth factor, a hormone, a fusion protein, an antibody or a fragment thereof. A therapeutic protein refers to a protein that can be used or that is used in therapy.

The term "recombinant protein" means a protein produced by recombinant technics. Recombinant technics are well within the knowledge of the skilled person (see for instance Sambrook et al., 1989, and updates).

As used in the specification and claims, the term "antibody", and its plural form "antibodies", includes, inter alia, polyclonal antibodies, affinity-purified polyclonal antibodies, monoclonal antibodies, and antigen-binding fragments, such as F(ab')2, Fab proteolytic fragments, and single chain variable region fragments (scFvs). Genetically engineered intact antibodies or fragments, such as chimeric antibodies, scFv and Fab fragments, as well as synthetic antigen-binding peptides and polypeptides, are also included.

The term "humanized" immunoglobulin refers to an immunoglobulin comprising a human framework region and one or more CDRs from a non-human (usually a mouse or rat) immunoglobulin. The non-human immunoglobulin providing the CDRs is called the "donor" and the human immunoglobulin providing the framework is called the "acceptor" (humanization by grafting non-human CDRs onto human framework and constant regions, or by incorporating the entire non-human variable domains onto human constant regions (chimerization)). Constant regions need not be present, but if they are, they must be substantially identical to human immunoglobulin constant regions, i.e., at least about 85-90%, preferably about 95% or more identical. Hence, all parts of a humanized immunoglobulin, except possibly the CDRs and a few residues in the heavy chain constant region if modulation of the effector functions is needed, are substantially identical to corresponding parts of natural human immunoglobulin sequences. Through humanizing antibodies, biological half-life may be increased, and the potential for adverse immune reactions upon administration to humans is reduced.

As used in the specification and claims, the term "fully human" immunoglobulin refers to an immunoglobulin comprising both a human framework region and human CDRs. Constant regions need not be present, but if they are, they must be substantially identical to human immunoglobulin constant regions, i.e., at least about 85-90%, preferably about 95% or more identical. Hence, all parts of a fully human immunoglobulin, except possibly few residues in the heavy chain constant region if modulation of the effector functions or pharmacokinetic properties are needed, are substantially identical to corresponding parts of natural human immunoglobulin sequences. In some instances, amino acid mutations may be introduced within the CDRs, the framework regions or the constant region, in order to improve the binding affinity and/or to reduce the immunogenicity and/or to improve the biochemical/biophysical properties of the antibody.

The term "recombinant antibodies" means antibodies produced by recombinant technics. Because of the relevance of recombinant DNA techniques in the generation of antibodies, one needs not be confined to the sequences of amino acids found in natural antibodies; antibodies can be redesigned to obtain desired characteristics. The possible variations are many and range from the changing of just one or a few amino acids to the complete redesign of, for example, the variable domain or constant region. Changes in the constant region will, in general, be made in order to improve, reduce or alter characteristics, such as complement fixation (e.g. complement dependent cytotoxicity, CDC), interaction with Fc receptors, and other effector functions (e.g. antibody dependent cellular cytotoxicity, ADCC), pharmacokinetic properties (e.g. binding to the neonatal Fc receptor; FcRn). Changes in the variable domain will be made in order to improve the antigen binding characteristics. In addition to antibodies, immunoglobulins may exist in a variety of other forms including, for example, single-chain or Fv, Fab, and (Fab')2 , as well as diabodies, linear antibodies, multivalent or multispecific hybrid antibodies.

As used herein, the term “antibody portion” refers to a fragment of an intact or a full-lenth chain or antibody, usually the binding or variable region. Said portions, or fragments, should maintain at least one activity of the intact chain / antibody, i.e. they are “functional portions” or “functional fragments”. Should they maintain at least one activity, they preferably maintain the target binding property. Examples of antibody portions (or antibody fragments) include, but are not limited to, "single-chain Fv”, "single-chain antibodies," "Fv" or "scFv". These terms refer to antibody fragments that comprise the variable domains from both the heavy and light chains, but lack the constant regions, all within a single polypeptide chain. Generally, a single-chain antibody further comprises a polypeptide linker between the VH and VL domains which enables it to form the desired structure that would allow for antigen binding. In specific embodiments, single-chain antibodies can also be bi-specific and/or humanized.

A "Fab fragment" is comprised of one light chain and the variable and CH1 domains of one heavy chain. The heavy chain of a Fab molecule cannot form a disulfide bond with another heavy chain molecule. A "Fab' fragment" that contains one light chain and one heavy chain and contains more of the constant region, between the CH1 and CH2 domains, such that an interchain disulfide bond can be formed between two heavy chains is called a F(ab')2 molecule. A "F(ab')2" contains two light chains and two heavy chains containing a portion of the constant region between the CH1 and CH2 domains, such that an interchain disulfide bond is formed between two heavy chains. Having defined some important terms, it is now possible to focus the attention on particular embodiments of the instant invention.

Examples of known antibodies which can be produced according to the present invention include, but are not limited to, adalimumab, alemtuzumab, belimumab, bevacizumab, canakinumab, certolizumab pegol, cetuximab, denosumab, eculizumab, golimumab, infliximab, natalizumab, ofatumumab, omalizumab, pertuzumab, ranibizumab, rituximab, siltuximab, tocilizumab, trastuzumab, ustekinumab or vedolizomab.

Most naturally occurring 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 predominant sugars found on glycoproteins are fucose, galactose, glucose, mannose, N-acetylgalactosamine ("GalNAc"), N-acetylglucosamine ("GlcNAc"), xylose and sialic acid. The oligosaccharide structure attached to the peptide chain is known as a "glycan" molecule. The nature of glycans impact the tridimensional structure and the stability of the proteins on which they are attached. The glycan structures found in naturally occurring glycopeptides are divided into two main classes: "N-linked glycans" or N-linked oligosaccharides" (main form in eukaryotic cells) and "O-linked glycans" or O-linked oligosaccharides". Peptides expressed in eukaryotic cells typically comprise N-glycans. The processing of the sugar groups for N-linked glycoproteins occurs in the lumen of the endoplasmic reticulum (ER) and continues in the Golgi apparatus. These N-linked glycosylations occur on asparagine residue in the peptide primary structure, on sites containing the amino acid sequence asparagine-X-serine/threonine (X is any amino acid residue except proline and aspartic acid).

Main glycans that can be found on the antibody or fragments thereof secreted by CHO cells are presented in Table 1:

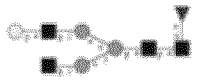
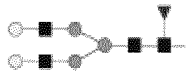
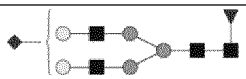
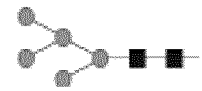
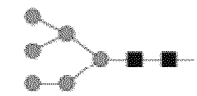
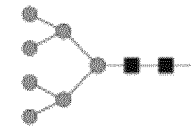
Glycan name	Glycan structure
G0	
G0F	
G1	
G1F	
G1F	
G2F	
G2F sialylated	
Man5	
Man6	
Man7	

Table 1 - main glycan structures (legend: grey squares: GlcNAc; mid-grey circles: mannose, light-grey circles: galactose; grey triangles: fucose; grey diamond: sialic acid)

"Glycoform" refers to an isoform of a protein, such as an antibody or a fragment thereof, differing only in the number and/or type of attached glycans. Usually, a composition comprising a glycoprotein comprises a number of different glycoforms of said glycoprotein.

Techniques for the determination of glycan primary structure are well known in the art and are described in detail, for example, in Roth et al. (2012) or Song et al. (2014). It is routine to isolate proteins produced by a cell and to determine the structure(s) of their N-glycans. N-glycans differ with respect to the number of branches (also called "antennae") comprising sugars, as well as in the nature of said branch(es), which can include in addition to the man3GlcNac2 core structure for instance N-acetylglucosamine, galactose, N-acetylgalactosamine, N-acetylneuraminic acid, fucose and/or sialic acid. For a review of standard glycobiology nomenclature see Essentials of Glycobiology, 1999.

Fucosylated proteins comprise at least one residue of fucose and include for instance glycans such as G0F, G1F and/or G2F (see above Table 1).

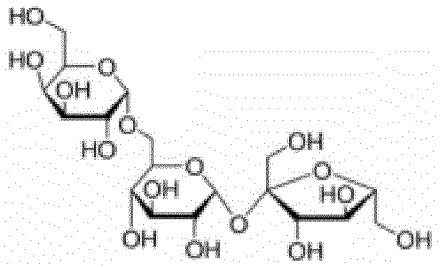
The N-glycans structures on proteins comprise at least three residues of mannose. These structures can be further mannosylated. The mannosylated glycans such as Man5, Man6 or Man7 are called high-mannose glycans (see above Table 1).

The term "subject" is intended to include (but not limited to) mammals such as humans, dogs, cows, horses, sheep, goats, cats, mice, rabbits, or rats. More preferably, the subject is a human.

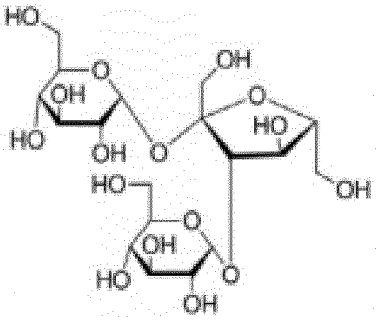
The present invention provides methods and compositions for modulating the glycosylation profile of a recombinant protein such as therapeutic protein or antibody. The present invention is based on the optimization of cell culture conditions for protein manufacturing, such as production of antibodies or antigen-binding fragments, resulting in the production of a recombinant protein with modulated glycosylation profiles, preferably with decreased fucosylation and/or increased mannosylation (i.e. an increase in high-mannose glycans, such as Man5).

It was observed that under cell culture conditions supplemented with a trisaccharide, the high mannosylated glycoform content of the recombinant protein increased and/or the fucosylated glycoform of the recombinant protein decreased. Thus during the cell culture production run, when it is desirable to modulate glycosylation profile of a recombinant protein, such as a fucosylation level and/or a mannosylation level in the recombinant protein being produced, the cell culture can be fed with a cell culture medium supplemented with a trisaccharide, such as D-(+)-Raffinose (raffinose herein), D-(+)-Melezitose (melezitose herein) and 1-Kestose (kestose herein). Alternatively, the cell culture medium can already comprise said trisaccharide. It was also observed that under cell culture conditions supplemented with a trisaccharide, higher titers could be achieved.

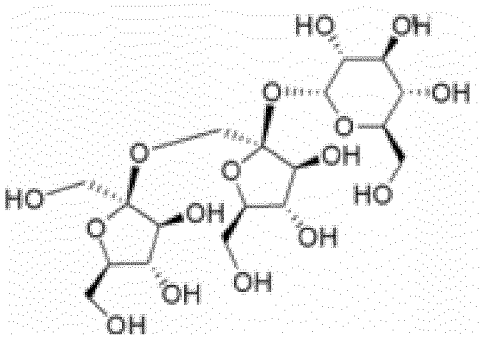
D-(+)-Raffinose : (O-α-D-Galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside)



D-(+)-Melezitose : O-α-D-Glucopyranosyl-(1→3)-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside



1-Kestose : β-D-Fructofuranosyl-(2→1)-β-D-Fructofuranosyl-(2→1)-α-D-glucopyranoside



In one aspect the invention provides a method of producing a recombinant protein with a modulated glycosylation profile, said method comprising culturing a recombinant cell expressing said protein in cell culture medium comprising or supplemented with a trisaccharide, such as raffinose, melezitose and kestose. In some preferred embodiments, the trisaccharide is raffinose.

Alternatively, the present invention describes a method of producing a recombinant protein with a modulated glycosylation profile, said method comprising culturing a host cell expressing said protein in cell culture medium complemented with at least one feed comprising a trisaccharide, such as raffinose, melezitose and kestose. In some preferred embodiments, the trisaccharide is raffinose.

In an embodiment, here is provided the use of a trisaccharide in a cell culture medium for modulating the glycosylation profile of recombinant proteins produced in mammalian cells, wherein the trisaccharide is for instance raffinose, melezitose or kestose. In a preferred embodiment, the trisaccharide is raffinose.

In a further aspect the invention provides a composition comprising a cell culture medium comprising a trisaccharide, such as raffinose, melezitose and kestose. In a preferred embodiment, the trisaccharide is raffinose.

In a further aspect the invention provides use of a trisaccharide, such as raffinose, melezitose and kestose for modulating the glycosylation profile of recombinant proteins. In some preferred embodiments of the foregoing, the trisaccharide is raffinose.

Preferably, in the context of the invention as a whole, the modulated glycosylation profile of the protein comprises modulation of the fucosylation level and/or of the mannosylation level in said protein. In particular, the modulation of the fucosylation level is a decrease in the overall fucosylation level in the recombinant protein and/or the modulation of the mannosylation level is an increase in the overall mannosylation level in the recombinant protein. More particularly the decrease in fucosylation level is due at least to a decrease in G0F and/or G1F forms, even more particularly the decrease in fucosylation level is due at least to a decrease in G0F form. More particularly the increase in mannosylation level is due at least to an increase in high-mannose forms, such as Man5. Preferably, the overall fucosylation level is decreased by about 0.1% to about 99% such as about 0.1 %, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 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%. Should the fucosyl residues completely disappear, the protein will be afucosylated. In another embodiment, the overall mannosylation amount or level is increased by about 0.1% to about 100% such as about 0.1 %, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 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%, 99% or 100%. Alternatively both modifications occur at the same time, i.e decrease of fucosylation and increase of mannosylation. As per the definition section, the modulation of the glycosylation level (such as the modulation of the fucosylation level and/or of the mannosylation level) is expressed in relation to the glycosylation level (e.g. the fucosylation level and/or of the mannosylation level) of the same protein produced by culturing a recombinant cell expressing said recombinant protein in cell culture media which is not supplemented with a trisaccharide.

The recombinant protein to be produced, in the context of the present invention as a whole, can be a therapeutic protein, an antibody or antigen binding fragment thereof, such as a human antibody or antigen-binding portion thereof, a humanized antibody or antigen-binding portion thereof, a chimeric antibody or antigen-binding portion thereof. Preferably, it is an antibody or antigen binding fragment thereof.

The methods of the present invention can be used to produce a protein, such as an antibody, having decreased amounts or levels of fucosyl residues and/or increased amounts or levels of mannosyl residues. Antibodies with such modified glycosylation profiles have been demonstrated to have an increased ADCC.

In the context of the invention as a whole, the trisaccharide compound, such as raffinose, melezitose and kestose is preferably present in a cell culture medium or added to a cell culture medium (as a supplement or as a feed) at a concentration of or of about 0.001 to 100 mM, even preferably at a concentration of or of about 0.01 to 50 mM, such as at concentration of or of about 0.001, 0.01, 0.05, 0.1, 0.5, 1, 2, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 or 100 mM (concentration of trisaccharide once in the culture medium in the culture system). For example, but not by way of limitation, by adjusting the concentration of a trisaccharide the glycosylation profile can be modulated.

For the purposes of this invention, cell culture medium is a medium suitable for growth of animal cells, such as mammalian cells, in *in vitro* cell culture. Cell culture media formulations are well known in the art. Cell culture media may be supplemented with additional components such as amino acids, salts, sugars, vitamins, hormones, and growth factors, depending on the needs of the cells in culture. Preferably, the cell culture media are free of animal components; they can be serum- free and/or protein- free.

In certain embodiments of the present invention, the cell culture medium is supplemented with the trisaccharide, for example, at the start of culture, and/or in a fed-batch or in a continuous manner. The addition of the trisaccharide supplement may be based on measured intermediate glycosylation profiles.

In an embodiment of the present invention, the host cell is preferably a mammalian host cell (herein also refer to as a mammalian cell) including, but not limited to, HeLa, Cos, 3T3, myeloma cell lines (for instance NS0, SP2/0), and Chinese hamster ovary (CHO) cells. In a preferred embodiment, the host cell is Chinese Hamster Ovary (CHO) cells.

In the context of the invention as a whole, the recombinant cell, preferably mammalian cell, is grown in a culture system such as a bioreactor. The bioreactor is inoculated with viable cells in a culture medium comprising or supplemented with a trisaccharide. Preferably the culture medium is serum-free and/or protein-free. Once inoculated into the production bioreactor the recombinant cells undergo an exponential growth phase. The growth phase can be maintained using a fed-batch process with bolus feeds of a feed medium optionally supplemented with said trisaccharide. Preferably the feed medium is serum-free and/or protein-free. These supplemental bolus feeds typically begin shortly after the cells are inoculated into the bioreactor, at a time when it is anticipated or determined that the cell culture needs feeding. For example, supplemental feeds can begin on or about day 3 or 4 of the culture or a day or two earlier or later. The culture may receive two, three, or more bolus feeds during the growth phase. Any one of these bolus feeds can optionally be supplemented with the trisaccharide. The supplementation or the feed with the trisaccharide can be done at the start of the culture, in fed-batch, and/or in continuous manner. Preferably, neither the basal cell culture medium nor the bolus feed medium contain galactose or sucrose. The culture medium can comprise glucose or be supplemented by glucose. Said supplementation can be done at the start of the culture, in fed-batch, and/or in continuous manner.

The methods, compositions and uses according to the present invention may be used to improve the production of recombinant proteins in multistep culture processes. In a multiple stage process, cells are cultured in two or more distinct phases. For example cells are cultured first in one or more growth phases, under conditions improving cell proliferation and viability, then transferred to production phase(s), under conditions improving protein production. In a multistep culture process, some conditions may change from one step (or one phase) to the other: media composition, shift of pH, shift of temperature, etc. The growth phase can be performed at a temperature higher than in production phase. For example, the growth phase can be performed at a first temperature from about 35°C to about 38°C, and then the temperature is shifted for the production phase to a second temperature from about 29°C to about 37°C. The cell cultures can be maintained in production phase for days or even weeks before harvest.

The cell lines (also referred to as "recombinant cells") used in the invention are genetically engineered to express a protein of commercial or scientific interest. Methods and vectors for genetically engineering of cells and/or cell lines to express a polypeptide of interest are well known to those of skill in the art; for example, various techniques are illustrated in Ausubel et al. (1988, and updates) or Sambrook et al. (1989, and updates). The methods of the invention can be used to culture cells that express recombinant proteins of interest. The recombinant proteins are usually secreted into the culture medium from which they can be recovered. The recovered proteins can then be purified, or partially purified using known processes and products available from commercial vendors. The purified proteins can then be formulated as pharmaceutical compositions. Suitable formulations for pharmaceutical compositions include those described in Remington's Pharmaceutical Sciences, 1995.

In a further aspect, the invention provides a composition comprising a recombinant protein with a modulated glycosylation profile produced by the methods of the invention.

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

In a further aspect, the invention provides a pharmaceutical composition comprising the recombinant protein with a modulated glycosylation profile produced by the methods of the invention and a pharmaceutically acceptable carrier. The recombinant protein is preferably a therapeutic protein, and can be an antibody or antigen binding fragment thereof, such as a human antibody or antigen-binding portion thereof, a humanized antibody or antigen-binding portion thereof, a chimeric antibody or antigen-binding portion thereof. Preferably, it is an antibody or antigen binding fragment thereof, with a decreased fucosylation level or amount and/or an increased mannosylation level or amount.

In certain embodiments, the pharmaceutical compositions of the invention comprising a recombinant protein with a modulated glycosylation profile 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 (see for instance Remington's Pharmaceutical Sciences, 1995). Such pharmaceutical compositions may comprise any one of salts, buffering agents, surfactants, solubilizers, polyols, amino acids, preservatives, compatible carriers, optionally other therapeutic agents, and combinations thereof. The pharmaceutical compositions of the invention comprising a recombinant protein with a modulated glycosylation profile, are present in a form known in the art and acceptable for therapeutic uses, such as liquid formulation, or lyophilized formulation. Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications without departing from the spirit or essential characteristics thereof. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

The present disclosure is therefore to be considered as in all aspects illustrated and not restrictive, the scope of the invention being indicated by the appended Claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.

The foregoing description will be more fully understood with reference to the following examples. Such Examples, are, however, exemplary of methods of practising the present invention and are not intended to limit the scope of the invention.

EXAMPLES

Material and methods

I. Cells, cell expansion and cell growth

1) Cells

Assays were performed with 2 CHO cell lines :

- CHO-S cells expressing IgG1 mAb1, herein “Cells mAb1” or “mAb1 cells”. “mAb1” is a fully human monoclonal antibody directed against a soluble protein. Its isoelectric point (pI) is about 8.20-8.30.
- CHO-K1 cells expressing IgG1 mAb2, herein “Cells mAb2” or “mAb2 cells”.“mAb2” is a humanized monoclonal antibody directed against a receptor found on the cell membrane. Its isoelectric point (pI) is about 9.30.

2) Cell expansion

Cell expansion was performed in tubes in a medium suitable for cell expansion. Assays in fed-batch started after at least one week expansion.

3) Inoculation

Cells expressing mAb2 were inoculated at 0.2 x 10^6 cells per millilitre (mL), whereas cells expressing mAb1 were inoculated at 0.3 x 10^6 cells per mL.

4) Fed-batch

All assays were performed in fed-batch culture.

A serum-free chemically defined culture medium was used. It was used as it is, or it was supplemented with D-(+)-Raffinose pentahydrate (Sigma-Aldrich, 83400-25G) at different concentrations (0-45mM). The concentrations indicated on figures 1, 2, 3 and 4 are the concentrations in raffinose in the culture medium at day 0, just after the inoculation. The culture medium was fed, on a regular basis, with a chemically defined feed medium, as well as with glucose in order to keep said glucose level in the range of >0 to about 8 g/L (feeds at day 3, 5, 7, 10 and 12).

The cultures were performed in deepwell plates with a working volume of 450µL. They were incubated at 36.5°C, 5% de CO_2, 90% humidity and shaken at 320rpm. Each of the fed-batch culture lasted 14 days.

II. Analytical methods

Viable cell density and viability were measured with the Guava easyCyte® flow cytometer.

Antibody titers were measured with the fortéBIO Octet®.

Glycosylation profiles were established by capillary gel electrophoresis with laser-induced fluorescence (CGE-LIF). Groups of glycans were defined as thereafter in Table 2.

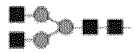
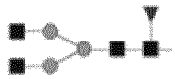
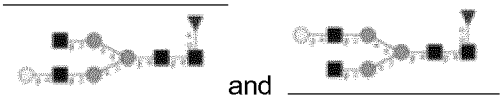
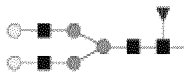
Group name	Composition
G0	
G0F	
G1F	 and
G2F	
Fuc	Fucosylated glycans
Gal	Galactosylated glycans
Man	High mannose glycans
Sial	Sialylated glycans
Ukn	Unknown glycans, not identified

Table 2 – Main groups of glycans identified (legend: grey squares: GlcNAc; mid-grey circles: mannose, light-grey circles: galactose; grey triangles: fucose)

Example 1 - Impact of raffinose on mAb1 antibody

The cells were cultivated and the results analysed as disclosed in the material and method section.

Viable cell density and viability:

Viable cell density and viability as a function of elapsed time, as well as antibody titer at the end of the fed-batch culture are shown on Figure 1. At concentrations lower than 8mM, raffinose allows increase of viable cell density comparing to control, whereas raffinose decreases growth at 25mM and at 42mM. At these concentrations, decrease of number of cells is reflected on titers, which decrease by 1.6 factor comparing to control. Impact on growth and titers is considered acceptable until about 10 to 15 mM for this antibody.

Glycosylation profiles:

Glycosylation profiles obtained by CGE-LIF analysis are shown on Figure 2. These data underline that raffinose is able to modulate the glycosylation of an antibody, and in particular is able to decrease fucosylated glycans and at the same time to increase mannosylated glycans. At 24.9mM for instance, fucosylation decreases by 3.1% and mannosylation increases by 2.6%.

Conclusion for mAb1:

At 10mM, a concentration at which cellular density and titers are satisfactory, raffinose allows decreasing fucosylation by 1.6% in overall and increasing mannosylation by 1.1% in overall. The main impact is seen on G0F form.

Example 2 - Impact of raffinose on mAb2 antibody

The cells were cultivated and the results analysed as disclosed in the material and method section.

Viable cell density and viability :

Viable cell density and viability as a function of elapsed time, as well as antibody titer at the end of the fed-batch culture are shown on Figure 3. These results show robustness of mAb2 cell line comparing to mAb1 cell line. Indeed, viability and titer decrease observed starting from 27mM is modest comparing to results obtained with mAb1 cells. The titer is for example decreased by 1.3 factor at 45mM, whereas it was 1.6 factor for mAb1 cells. On the other hand, high raffinose concentrations allow maintaining important viability at the end of cell culture.

Glycosylation profiles :

Glycosylation profiles of antibodies after 14 days of culture are shown on Figure 4. At about 27 mM raffinose, the proportion of mannosylated glycans increases by 5.8% in overall and percentage of fucosylated form decreases by 6.3% in overall. As for mAb1, the main impact is seen on G0F form.

Conclusion for mAb2:

At 25 mM, a concentration at which cellular density and titers are satisfactory, raffinose allows decreasing fucosylation by 6.3% and increasing mannosylation by 5.8%.

Overall conclusion:

Examples 1 and 2 show that raffinose allows modulating mannosylation and fucosylation of monoclonal antibodies, without having negative impact on cell viability and viable cell density. The skilled person will understand from the results of examples 1 and 2 that he can use raffinose for modulating the glycosylation profile of any antibodies and any proteins, whatever the cell line that is used for production, and in particular to decrease the overall fucosylation level and to increase the overall mannosylation level. The exact concentration of raffinose to be added in the cell culture media will have to be determined case by case, depending on the glycosylation profile the skilled one wish to obtain molecule per molecule. This determination can be done without involving any inventive skill, based on the teaching of the present invention. The skilled person will also understand that he can use raffinose in any method for producing a protein such as an antibody, even if he does not aim to reach a particular glycosylation profile.

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2) Yu et al., 2012. Characterization and pharmacokinetic properties of antibodies with N-linked Mannose-5 glycans"; mAbs, 4(4):475-487.

3) Cell Culture Technology for Pharmaceutical and Cell-Based Therapies, Sadettin Ozturk, Wei-Shou Hu, ed., CRC Press (2005)

4) Ziv Roth et al., 2012. Identification and Quantification of Protein Glycosylation; International Journal of Carbohydrate Chemistry, Article ID 640923.

5) Ting Song et al., 2014. In-Depth Method for the Characterization of Glycosylation in Manufactured Recombinant Monoclonal Antibody Drugs; *Anal. Chem.*, *86*(12):5661–5666

6) Voisard et al., 2003, Biotechnol. Bioeng. 82:751-765

7) Ausubel et al., 1988 and updates, Current Protocols in Molecular Biology, eds. Wiley & Sons, New York.

8) Sambrook et al., 1989 and updates, Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press.

9) Remington's Pharmaceutical Sciences, 1995, 18th ed., Mack Publishing Company, Easton, PA

CLAIMS

1. A method of producing a recombinant protein with a modulated glycosylation profile, said method comprising culturing a host cell expressing said protein in cell culture medium comprising a trisaccharide or supplemented with a trisaccharide.

2. A method of producing a recombinant protein with a modulated glycosylation profile, said method comprising culturing a host cell expressing said protein in cell culture medium complemented with at least one feed comprising a trisaccharide.

3. The method of claim 1 or claim 2, further comprising purifying said recombinant protein with a modulated glycosylation profile.

4. The method of any one of claims 1 to 3, wherein the modulated glycosylation profile of the protein comprises modulation of fucosylation level and/or mannosylation level in said protein.

5. The method of claim 4, wherein the modulation of the fucosylation level is a decrease in the fucosylation level and wherein the modulation of the mannosylation level is an increase in the mannosylation level in said protein.

6. The method of any one of claims 1 to 5, wherein the trisaccharide is raffinose.

7. The method of any one of claims 1 to 6, wherein the host cell is Chinese Hamster Ovary (CHO) cells.

8. A composition comprising a cell culture medium comprising a trisaccharide or supplemented with a trisaccharide.

9. The composition of claim 8, wherein the trisaccharide is raffinose.

10. A pharmaceutical composition comprising the recombinant protein with a modulated glycosylation profile produced by the methods of any one of claims 1 to 7 and a pharmaceutically acceptable carrier.

11. A composition comprising a recombinant protein with a modulated glycosylation profile produced by the methods of any one of claims 1 to 7.

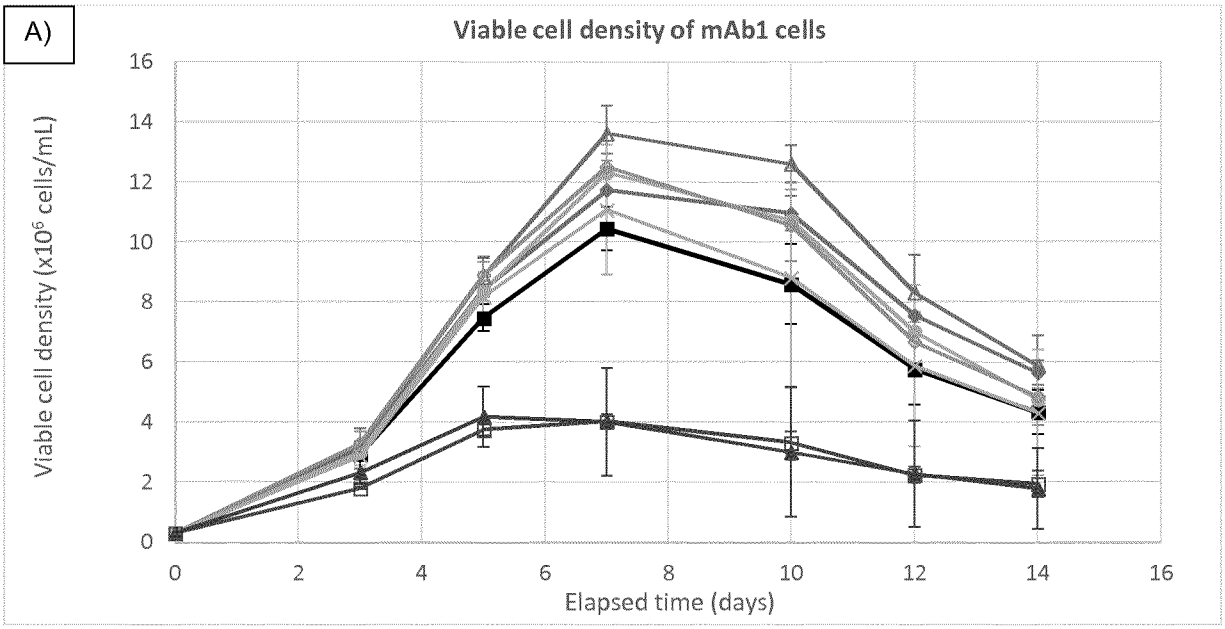
12. The method of any one of claims 1 to 7, the pharmaceutical composition of claim 10 or the composition of claim 11, wherein the recombinant protein is selected from the group consisting of an antibody or antigen binding fragment thereof, such as a human antibody or antigen-binding portion

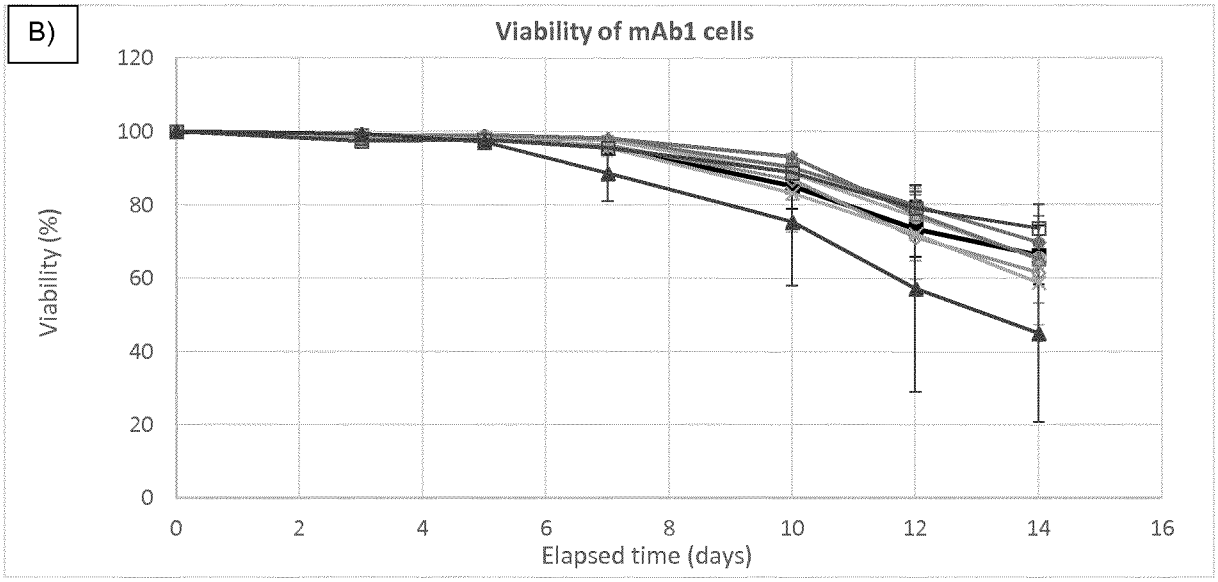
thereof, a humanized antibody or antigen-binding portion thereof, a chimeric antibody or antigen-binding portion thereof, a recombinant fusion protein, a growth factor, a hormone, or a cytokine.

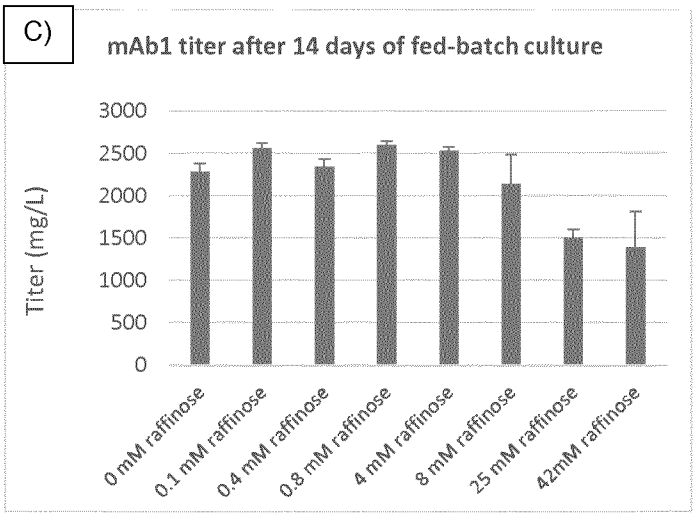
13. Use of a trisaccharide in a cell culture medium for modulating the glycosylation profile of recombinant proteins.

14. The use of claim 13, wherein the trisaccharide is raffinose and wherein the modulated glycosylation profile of the proteins comprises modulation of a fucosylation level and/or a mannosylation level in said proteins.

15. The method according to any one of claims 1 to 7 or 12, the composition according to any one of claims 8 and 9 or the use according to any one of claims 13 and 14, wherein the concentration of trisaccharide in the cell culture medium is of about 0.01mM to 50mM.


A)
Viable cell density of mAb1 cells
Viable cell density (x10^6 cells/mL)
Elapsed time (days)


B)
Viability of mAb1 cells
Viability (%)
Elapsed time (days)


C)
mAb1 titer after 14 days of fed-batch culture
Titer (mg/L)
0 mM raffinose
0.1 mM raffinose
0.4 mM raffinose
0.8 mM raffinose
4 mM raffinose
8 mM raffinose
25 mM raffinose
42mM raffinose

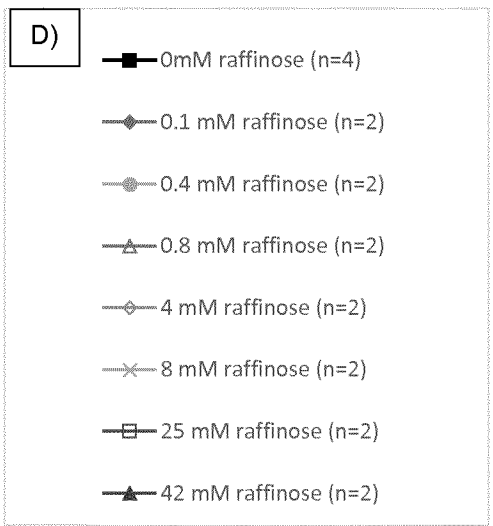

D)
0mM raffinose (n=4)
0.1 mM raffinose (n=2)
0.4 mM raffinose (n=2)
0.8 mM raffinose (n=2)
4 mM raffinose (n=2)
8 mM raffinose (n=2)
25 mM raffinose (n=2)
42 mM raffinose (n=2)

Figure 1

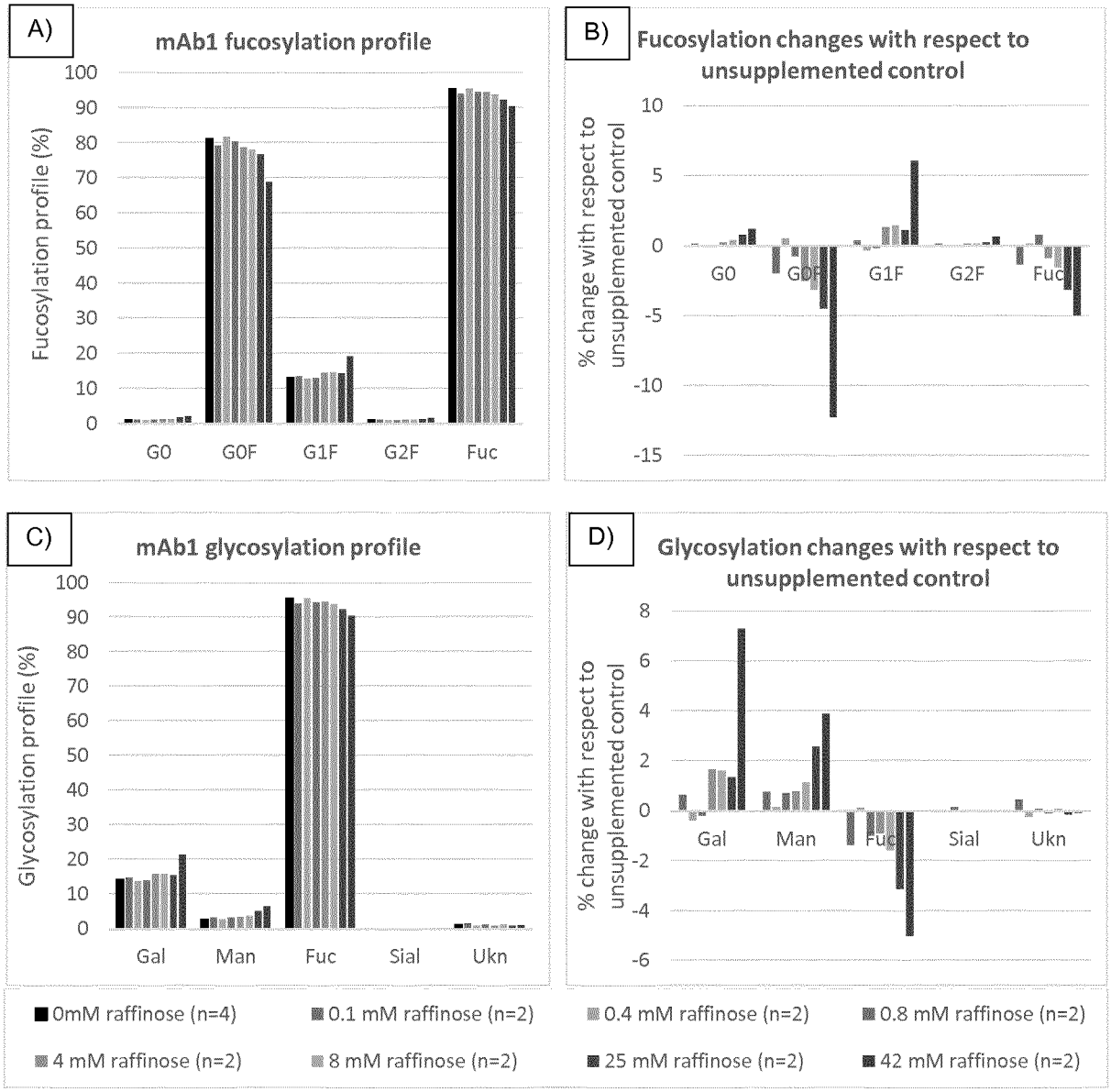
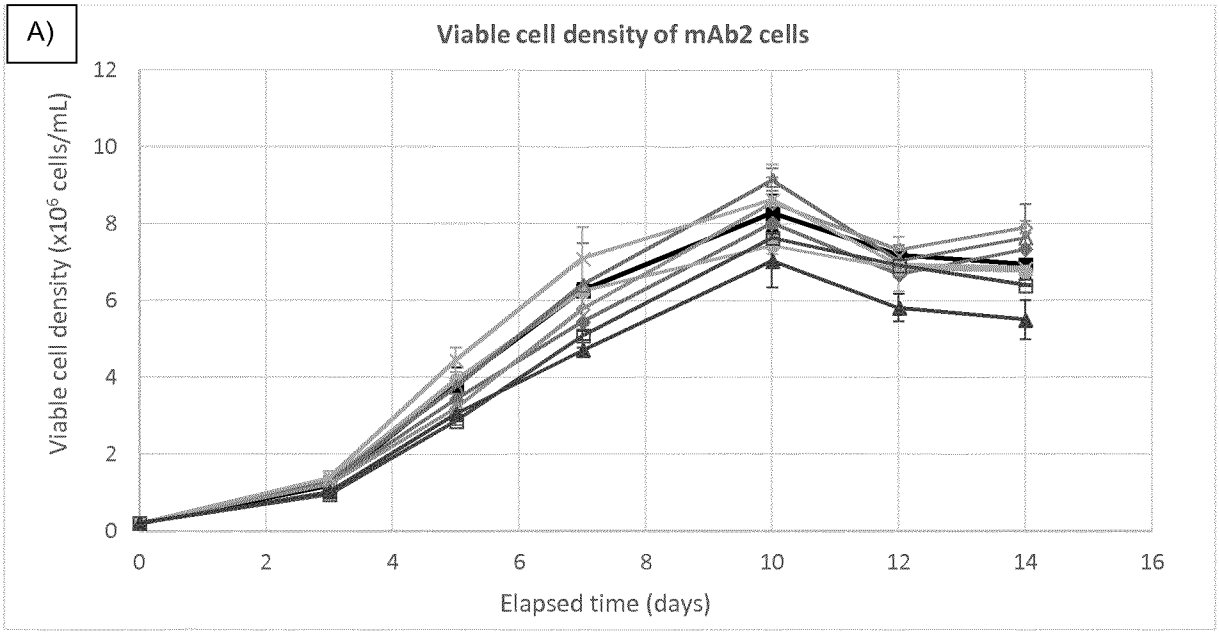
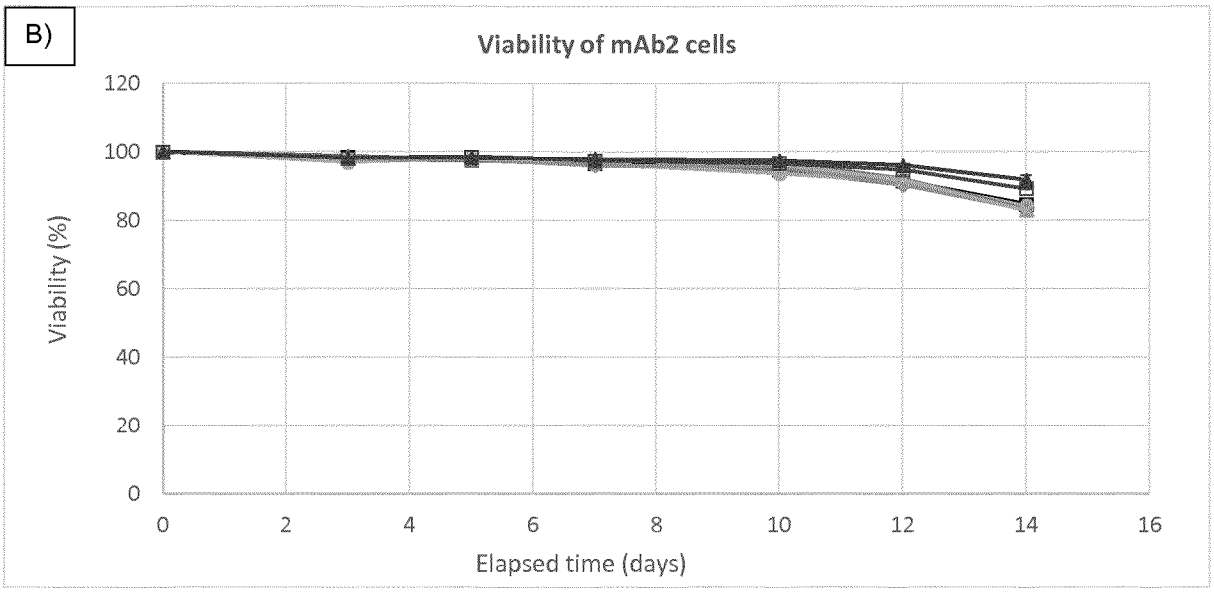
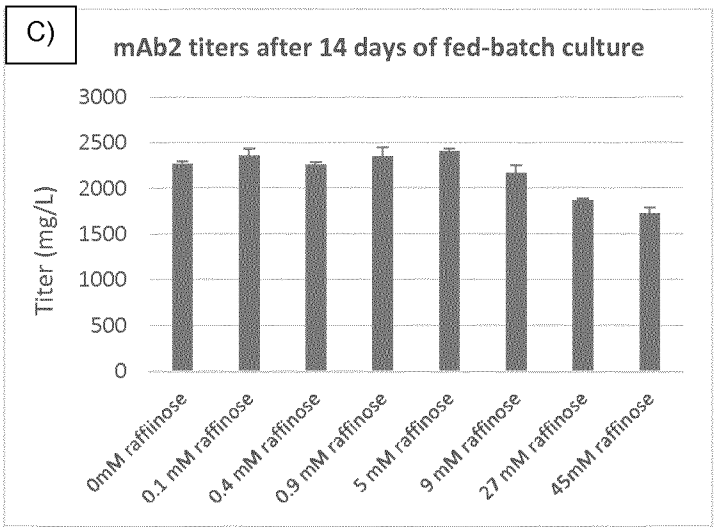

A)
mAb1 fucosylation profile
Fucosylation profile (%)
100
90
80
70
60
50
40
30
20
10
0
G0
G0F
G1F
G2F
Fuc
B)
Fucosylation changes with respect to unsupplemented control
% change with respect to unsupplemented control
10
5
0
-5
-10
-15
G0
G0F
G1F
G2F
Fuc
C)
mAb1 glycosylation profile
Glycosylation profile (%)
100
90
80
70
60
50
40
30
20
10
0
Gal
Man
Fuc
Sial
Ukn
D)
Glycosylation changes with respect to unsupplemented control
% change with respect to unsupplemented control
8
6
4
2
0
-2
-4
-6
Gal
Man
Fuc
Sial
Ukn
0mM raffinose (n=4)
0.1 mM raffinose (n=2)
0.4 mM raffinose (n=2)
0.8 mM raffinose (n=2)
4 mM raffinose (n=2)
8 mM raffinose (n=2)
25 mM raffinose (n=2)
42 mM raffinose (n=2)

Figure 2


A)
Viable cell density of mAb2 cells
Viable cell density (x10⁶ cells/mL)
Elapsed time (days)


B)
Viability of mAb2 cells
Viability (%)
Elapsed time (days)


C)
mAb2 titers after 14 days of fed-batch culture
Titer (mg/L)
0mM raffinose
0.1 mM raffinose
0.4 mM raffinose
0.9 mM raffinose
5 mM raffinose
9 mM raffinose
27 mM raffinose
45mM raffinose

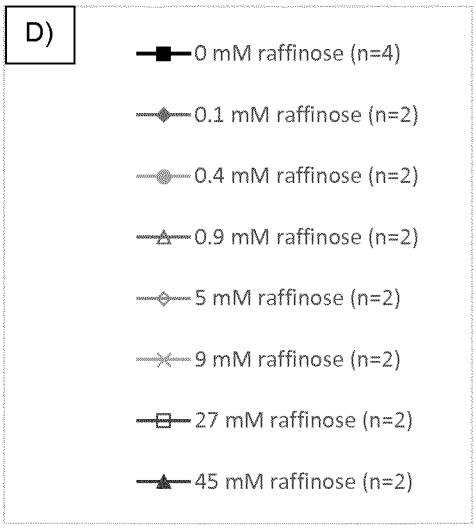

D)
0 mM raffinose (n=4)
0.1 mM raffinose (n=2)
0.4 mM raffinose (n=2)
0.9 mM raffinose (n=2)
5 mM raffinose (n=2)
9 mM raffinose (n=2)
27 mM raffinose (n=2)
45 mM raffinose (n=2)

Figure 3

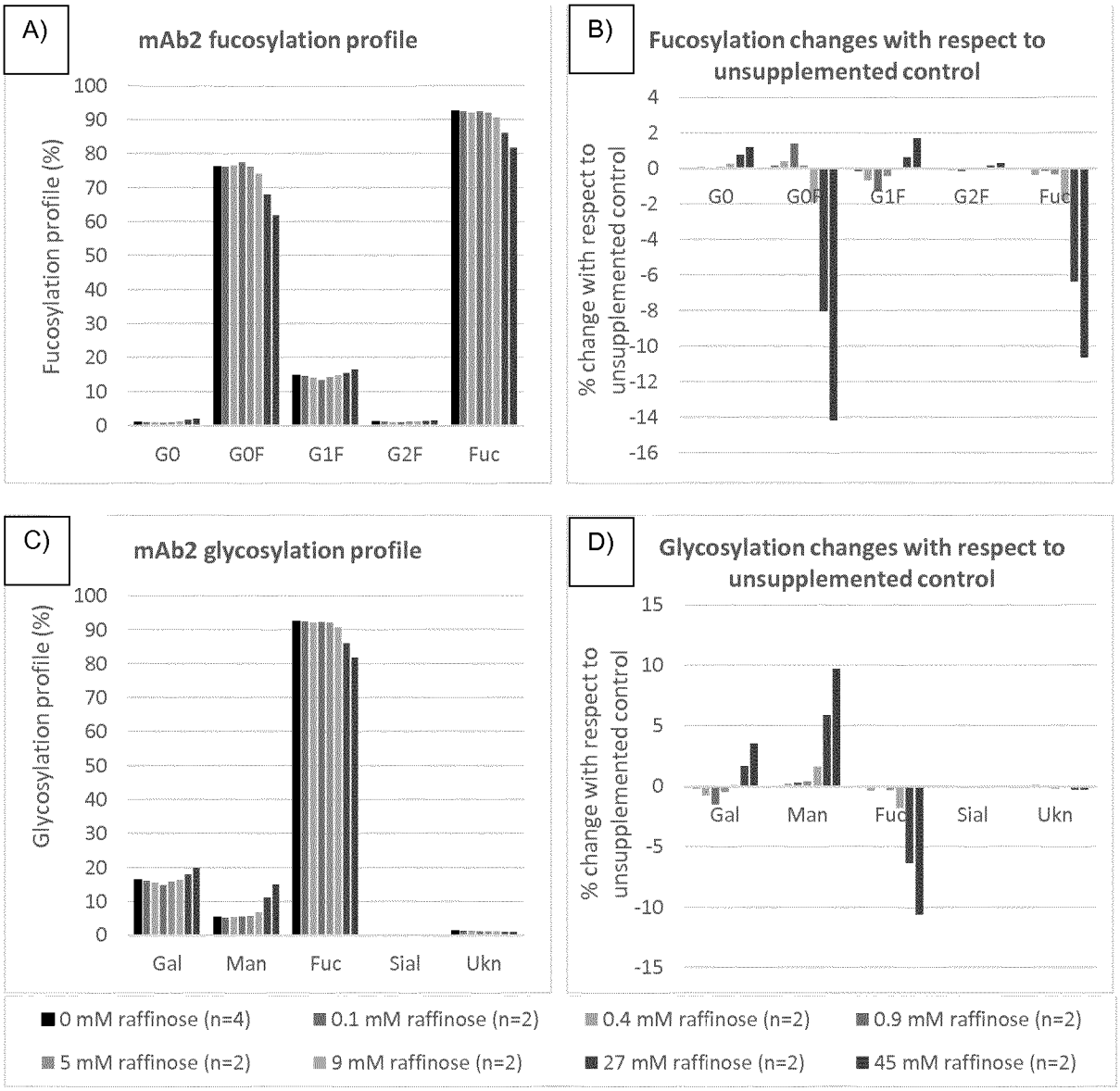

A)
mAb2 fucosylation profile
Fucosylation profile (%)
100
90
80
70
60
50
40
30
20
10
0
G0
G0F
G1F
G2F
Fuc
B)
Fucosylation changes with respect to unsupplemented control
% change with respect to unsupplemented control
4
2
0
-2
-4
-6
-8
-10
-12
-14
-16
G0
G0F
G1F
G2F
Fuc
C)
mAb2 glycosylation profile
Glycosylation profile (%)
100
90
80
70
60
50
40
30
20
10
0
Gal
Man
Fuc
Sial
Ukn
D)
Glycosylation changes with respect to unsupplemented control
% change with respect to unsupplemented control
15
10
5
0
-5
-10
-15
Gal
Man
Fuc
Sial
Ukn
0 mM raffinose (n=4)
0.1 mM raffinose (n=2)
0.4 mM raffinose (n=2)
0.9 mM raffinose (n=2)
5 mM raffinose (n=2)
9 mM raffinose (n=2)
27 mM raffinose (n=2)
45 mM raffinose (n=2)

Figure 4

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/057818

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015/032413 A1 (GLYCOM AS [DK]) 12 March 2015 (2015-03-12) abstract -----	8
X	EP 2 409 989 A1 (INT DRUG DEV BIOTECH [FR]) 25 January 2012 (2012-01-25) abstract; page 16, line 15 - page 18, line 33 -----	10,11
A	WO 2012/140138 A1 (LEK PHARMACEUTICALS [SI]; KONCILJA MATJAZ [SI]; SPUDIC VATROSLAV [SI];) 18 October 2012 (2012-10-18) page 2, paragraph 1 - page 4, paragraph 3 -----	1-15
A	WO 2015/026846 A1 (BIOGEN IDEC INC [US]) 26 February 2015 (2015-02-26) paragraph [0222] - paragraph [0246] -----	1-15
	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 May 2016	07/06/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Kalsner, Inge

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2014/271632 A1 (HOSSLER PATRICK [US] ET AL) 18 September 2014 (2014-09-18) paragraph [0006] - paragraph [0024]	1-15
A	MARCELLA YU ET AL: "Production, characterization and pharmacokinetic properties of antibodies with N-linked Mannose-5 glycans", MABS, vol. 4, no. 4, 1 July 2012 (2012-07-01), pages 475-487, XP055168632, ISSN: 1942-0862, DOI: 10.4161/mabs.20737 abstract	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
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		EP 2596015 A1	29-05-2013
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WO 2015026846 A1	26-02-2015	NONE	
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