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(71) Applicant: **IMPERIAL COLLEGE INNOVATIONS LIMITED** [GB/GB]; Level 1 Faculty Building, c/o Imperial College, Exhibition Road, London SW7 2AZ (GB).

(72) Inventors: **MAKRYDAKI, Elli**; c/o Imperial College Innovations Limited, Level 1 Faculty Building, c/o Imperial College, Exhibition Road, London SW7 2AZ (GB). **KONTORAVDI, Cleo**; c/o Imperial College Innovations Limited, Level 1 Faculty Building, c/o Imperial College, Exhibition Road, London SW7 2AZ (GB). **POLIZZI, Karen**; c/o Imperial College Innovations Limited, Level 1 Faculty Building, c/o Imperial College, Exhibition Road, London SW7 2AZ (GB). **HASLAM, Stuart**; c/o Imperial College Innovations Limited, Level 1 Faculty Building, c/o Imperial College, Exhibition Road, London SW7 2AZ (GB). **MOYA-RAMIREZ, Ignacio**; c/o Imperial College Innovations Limited, Level 1 Faculty Building, c/o Imperial College, Exhibition Road, London SW7 2AZ (GB). **ROYLE, Kate**; c/o Imperial College Innovations Limited, Level 1 Faculty Building, c/o Imperial College, Exhibition Road, London SW7 2AZ (GB).

(74) Agent: **LAU, Sarah**; Lacon London, 84 Theobalds Road, LONDON WC1X 8NL (GB).

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(54) Title: METHODS AND POLYNUCLEOTIDES

(57) Abstract: The present invention provides an *in vitro* method of modifying the glycosylation pattern of a glycoprotein, comprising contacting said glycoprotein or a glycan component of said glycoprotein with an immobilised and biotinylated enzyme that is involved in a glycosylation pathway. The invention also provides polynucleotides, vectors, host cells and polypeptides related to said method. The invention further provides an *in vitro* method of producing an immobilised and biotinylated enzyme that is involved in a glycosylation pathway, comprising the steps of: (a) co-expressing a polynucleotide encoding a solubility tag, a catalytic domain of said enzyme, a linker and an AviTag, as defined herein, with a polynucleotide encoding the enzyme BirA; (b) adding biotin; and (c) contacting the expressed polypeptide with streptavidin or avidin.

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METHODS AND POLYNUCLEOTIDES

Field of the invention

The present invention relates to an *in vitro* method of modifying the glycosylation pattern of a glycoprotein using an immobilised and biotinylated enzyme that is involved in a glycosylation pathway. The invention also relates to a polynucleotide construct that is of use in the production of such an immobilised and biotinylated enzyme. The method of the invention can be used to modify a glycoprotein or a glycan component of a glycoprotein *in vitro*, for example in a sequential glycosylation reaction that avoids the heterogeneity associated with known methods. The present invention also relates to a vector and host cell comprising the polynucleotide construct, and a polypeptide encoded by the polynucleotide construct.

Background to the invention

N-linked glycosylation is one of the most important post-translational modifications of proteins. It is a key quality attribute of biotherapeutics as it can affect drug efficiency, efficacy and half-life. Naturally, glycosylation is a non-templated and complex process owing firstly to the promiscuity of the enzymes involved and secondly to the variability of enzyme expression levels. This leads to natural heterogeneity of cell-derived glycoproteins which makes it very difficult to understand the role of individual glycoforms in biological processes and it can complicate the large-scale and bespoke application of proteins for use as therapeutics, vaccines, or materials.

The dominating approach to address heterogeneity is cell-line engineering of host cells. Engineering cells for bespoke functions can interfere with their needs for critical processes, making it difficult to scale-up production, resulting in an expensive and time-consuming approach. In addition, there are several *in vitro* techniques aiming to control glycosylation. Chemoenzymatic glycosylation is an example, where a precursor substrate is further modified by enzymes or by chemical conjugation (Overkleeft, H. S. & Seeberger, P. H. *Chemoenzymatic Synthesis of Glycans and Glycoconjugates. Essentials of Glycobiology* (Cold Spring Harbor Laboratory Press, 2015)). There is also the use of enzymes for *in vitro* modifications of glycans deriving from proteins in a one-pot fashion (Hamilton, B. S. *et al.* A Library of Chemically Defined Human N-Glycans Synthesized from Microbial Oligosaccharide Precursors. *Sci. Rep.* **7**, (2017)). These methods are limited due to the difficult and complicated implementation or the lack of control over the enzyme promiscuity, and as such costly intermediate purifications are necessary. This cost is further increased due to the inability to recycle and reuse the enzymes.

O-linked glycosylation, the post-translational addition of glycans to Serine/Theronine, contributes to various physiological process such as immunity and development. O-linked glycans serve as biomarkers for blood type identification as well as cancer development, while they serve as target for glycan binding proteins. Unlike its counterpart N-linked glycosylation, O-linked glycosylation is less complex. However, the lack of templated reactions, the enzyme promiscuity and enzyme availability can lead to multiple glycan structures and consequently heterogeneity (Reily, C. *et al.* Glycosylation in health and disease. *Nature Reviews Nephrology* (2019). doi:10.1038/s41581-019-0129-4; Kudelka, M. R. *et al.* Simple sugars to complex disease-mucin-type O-glycans in cancer. in *Advances in Cancer Research* (2015). doi:10.1016/bs.acr.2014.11.002).

Klymenko et al (*AIChE J.* **62**, 2959-2973, 2016) describe the considerations for designing an artificial

Golgi reactor to achieve targeted and sequential glycosylation reactions.

WO2006/102652 relates to methods of producing soluble, active eukaryotic glycosyltransferases in prokaryotic microorganisms that have an oxidising environment.

Summary of the invention

The present inventors have demonstrated the design and application of an artificial Golgi reactor, to build the desired N-linked glycosylation of glycoproteins by performing an immobilised enzyme cascade. The inventors have designed novel constructs for the expression of such immobilised enzymes and these also form part of the present invention. The spatiotemporal separation of enzymes in such a system allows high control over the enzyme promiscuity, leading to greater homogeneity. Furthermore, it is a cost-effective approach as it enables enzyme reusability. The system of immobilised enzymes can be used to tailor the glycosylation profile of glycosylated therapeutic proteins produced *in vivo* (e.g. in CHO, human cells or other cheaper production platforms such plants or yeast) or from cell-free protein synthesis systems. As this strategy is applied post-expression, it can be easily adapted to any glycosylation pathway by changing the enzymes used. The modularity of this system allows to test the biological role of different glycans whilst synthesising a range of glycosylated proteins. The system can also be used for O-linked glycosylation of proteins, for example therapeutic proteins.

In a first aspect, the present invention provides an *in vitro* method of modifying the glycosylation pattern of a glycoprotein, comprising contacting said glycoprotein or a glycan component of said glycoprotein with an immobilised and biotinylated enzyme that is involved in a glycosylation pathway.

Detailed description of the invention

The method of the first aspect of the invention is an *in vitro* method of modifying the glycosylation pattern of a glycoprotein. This may alternatively be worded as an *in vitro* method of altering the glycosylation profile of a glycoprotein. The method of the invention is used to change glycan structures that are covalently linked to a glycoprotein, by adding or removing one or more glycans using appropriate enzymes. The method comprises contacting said glycoprotein or a glycan component of said glycoprotein with an immobilised and biotinylated enzyme that is involved in a glycosylation pathway. The method can therefore be carried out either directly on a glycoprotein or alternatively on a glycan component which is subsequently added to a glycoprotein, for example during the preparation of glycoconjugate vaccines. Such vaccines are prepared by covalently linking a bacterial polysaccharide to a protein.

The method of the first aspect of the invention relates to the Artificial Golgi system described herein. One of the numerous advantages of such a system is that it enables the sequential glycosylation and/or deglycosylation of a glycoprotein or glycan component by multiple enzymes.

Accordingly, the glycoprotein or glycan component can be sequentially contacted with more than one immobilised and biotinylated enzyme that is involved in a glycosylation pathway.

The glycosylation pathway may be, for example, an N- or O-linked glycosylation pathway.

The enzyme is typically eukaryotic, for example from a human (*Homo sapiens*), an animal (such as a rabbit **(***Oryctolagus cuniculus)*, rat (*Rattus norvegicus)*, mouse (*Mus musculus)* chicken (*Gallus gallus*), cow (*Bos taurus*), goat (*Capra hircus),* zebrafish (*Danio rerio*), fruitfly (*Drosophila melanogaster)* or nematode worm (such as *Caenorhabditis elegans*)) or a plant (such as *Arabidopsis thaliana* or *Nicotiana tabacum*).

The enzyme may alternatively be of prokaryotic origin, for example from *Neisseria meningitis* or *Neisseria gonorrhoeae.*

The enzyme is typically a glycosyltransferase or a glycosidase. Glycosyltransferases catalyse the formation of the glycosidic linkage to form a glycoside. These enzymes utilize "activated" sugar phosphates as glycosyl donors, and catalyze glycosyl group transfer to a nucleophilic group, usually an alcohol. Glycosidases catalyse the hydrolysis of glycosidic linkages.

The enzyme is typically involved in a N-linked glycosylation pathway. Examples of suitable enzymes involved in N-linked glycosylation pathways include β-1,4 galactosyltransferase (GalT), *N*-acetylglucosaminyltransferase I (GnTI), *N*-acetylglucosaminyltransferase II (GnTII), *N*-acetylglucosaminyltransferase III (GnTIII), *N*-acetylglucosaminyltransferase IV (GnTIV), *N*-acetylglucosaminyltransferase V (GnTV) or sialyltransferase (SiaT) glycoprotein 6-alpha-L-fucosyltransferase (FucT) and α-mannosidase II (ManII). In an embodiment, the enzyme is GalT, GnTI or ManII. Figure 1a. shows the N-linked glycosylation pathway of GnTI, ManII and GalT,

Tables 1, 2 and 3 below respectively give examples of suitable GnTI, ManII and GalT enzymes for use in the present invention.

Table 1

GnTI enzymes

Enzyme	Expression system	Immobilisation	Ref.
***Homo sapiens* GnTI** (445 amino acids)	Human GnTI lacking first 103 amino acids (Δ103) in *E. coli* as an MBP fusion protein	Affinity immobilisation of MBP fusion on amylose beads	1
	MBP-Δ103 hGnTI in *E. coli* (Top 10 cells). Mutations of the unpaired C121 shown to have an impact on specific activity	N/A	2
	His-Δ103 hGnTI in *E. coli* (trxB/gor mutations strain).	N/A	3
	hGnTI with N-terminus viral tag epitope in Sf9 insect cells	N/A	4
	hGnTI-Myc in Lec1 CHO cells a with different transmembrane domains.	N/A	5
***Arabidopsis thaliana* GnTI** (444 amino Acids)	Δ24 AtGnTI-His in Sf21 insect cells using the Baculovirus expression system	N/A	6

***Oryctolagus cuniculus* (rabbit) GnTI** (447 amino acids)	Δ29 OcGnTI-His Sf21 insect cells using the Baculovirus expression system	N/A	[6]
***Rattus norvegicus* GnTI (**447 amino acids)	Δ37 RnGnTI *E. coli* (BL21 cells) as a His-tag fusion protein (N- or C- terminus)	N/A	[7]
***Drosophila melanogaster* GnTI** (458 amino acids)	His-Δ25 DmGnTI was expressed in Sf9 insect cells	N/A	[8]
Caenorhabditis elegans GnTI.2 Homologous to **GnTI** (449 amino acids)	*C. elegans* Gly-13 GnTI.2 was expressed as a His tag fusion protein in Sf9 insect cells	N/A	[9]
Nicotiana tabacum GnTI (446 amino acids)	MBP-Δ29 NtGnTI *E. coli* (Rosetta DE3-Gami) cells	N/A	[10]
	Δ29 NtGnTI- GST in Sf9 insect cells	N/A	[11]
	NtGnTI-MBP in various *E. coli* strains	N/A	[12]

Table 2

ManII enzymes

Enzyme	Expression system	Immobilisation	Ref.
***Homo sapiens* ManII** (1144 amino acids)	Protein A-hManII (only catalytic domain) in COS cells	Affinity immobilisation on IgG-agarose beads	13
***Homo sapiens* ManIIX (MX)** (1139 amino acids)	His_6-hMX (only catalytic domain) in CHO cells	N/A	14
	Protein A-hMX (only catalytic domain) in COS cells	Affinity immobilisation on IgG-agarose beads	13
***Arabidopsis thaliana* ManII** (1173 amino acids)	His-AtManII in Sf21 insect cells using the Baculovirus expression system	N/A	15
***Drosophila melanogaster* ManII** (1108 amino acids)	Overexpression in S2 insect cells via stable transfection Produced 60mg/ml of purified enzyme	N/A	16
	Protein A-Δ74 DmManII transiently expressed in CHOP cells	Affinity immobilisation on IgG-sepharose Beads	17
***Capra hircus* ManII** (1144) amino acids)	ChManII-His_6 was expressed in *P. pastoris*	N/A	18
***Caenorhabditis elegans* ManII** (1145 amino acids)	Δ122Ce ManII expressed in *P. pastoris*	N/A	19
***Mus musculus* ManII** (1150 amino acids)	MmManII was transiently expressed in COS cells	N/A	20

Table 3

GalT enzymes

Enzyme	Expression system	Immobilisation	Ref.
Homo sapiens **GalT** (398 amino acids)	hGalT was expressed in *S. cerevisiae* strain BT150 (only catalytic domain)	N/A	21
	hGalT was expressed in *E. coli*	N/A	22
	MBP-hGalT-LPETG-His_6 was expressed in *E. coli*	Sortase-mediated immobilisation (covalent)	23
	hGalT in *N. tabacum* BY2 cells	N/A	24
	His_6- *S. hyicus* lipase pre-propeptide- hGalT (only catalytic domain)	Affinity immobilisation on Ni/NTa magnetic beads	25
	hGalT-MBP in *E. coli*	Covalent immobilisation on CNBr-activated Sepharose beads	26
	MBP-hGalT (lacking transmembrane domain) in *E. coli*	N/A	27
Gallus Gallus **(chicken) GalT** (362 amino acids) ***Danio rerio*** **(zebrafish) GalT** (350amino acids)	GgGalT Beta-1,4-galactosyltransferase 1 was fused to N-terminal fragments of human GalT or rat ST6Gal1 and expressed in *N. tabacum* leaves	N/A	28
Bos Taurus **(bovine) GalT** (402 amino acids)	BtGalT lacking the cytosolic and transmembrane domain (CTD), was N-terminally fused to the CTD of FuT7 and an 8XHis tag while expressed in *Sf9* cells.	N/A	29

Neisseria meningitis (275 amino acids) **& *Neisseria gonorrhoeae*** (275 amino acids) **GalT**	GalT-His_6 tag to C-terminus was expressed in *E. coli* BL21 cells	N/A	30

1. Fujiyama, K. *et al.* Human N-Acetylglucosaminyltransferase I. Expression in Escherichia coli as a Soluble Enzyme, and Application as an Immobilized Enzyme for the Chemoenzymatic Synthesis of N-Linked Oligosaccharides. *J. Biosci. Bioeng.* **92**, 569–574 (2001).

2. Saribas, A. S., Johnson, K., Liu, L., Bezila, D. & Hakes, D. Refolding of human ??-1-2 GlcNAc transferase (GnT1) and the role of its unpaired Cys 121. *Biochem. Biophys. Res. Commun.* **362**, 381–386 (2007).

3. Chen, R., Pawlicki, M. A., Hamilton, B. S. & Tolbert, T. J. Enzyme-Catalyzed Synthesis of a Hybrid N-Linked Oligosaccharide using N-Acetylglucosaminyltransferase I. *Adv. Synth. Catal.* **350**, 1689–1695 (2008).

4. Wagner, R. *et al.* Elongation of the N-glycans of fowl plague virus hemagglutinin expressed in Spodoptera frugiperda (Sf9) cells by coexpression of human ??1,2-N-acetylglucosaminyltransferase I. *Glycobiology* **6**, 165–175 (1996).

5. Opat, A. S., Houghton, F. & Gleeson, P. A. Medial Golgi but not Late Golgi Glycosyltransferases Exist as High Molecular Weight Complexes. Role of Luminal Domain in Complex Formation and Localization. *J. Biol. Chem.* **275**, 11836–45 (2000).

6. Strasser, R. *et al.* Molecular basis of N-acetylglucosaminyltransferase I deficiency in Arabidopsis thaliana plants lacking complex N-glycans. *Biochem. J.* **387**, 385–391 (2005).

7. Nishiu, J., Kioka, N., Fukada, T., Sakai, H. & Komano, T. Characterization of rat N-acetylglucosaminyltransferase I expressed in Escherichia coli. *Biosci. Biotechnol. Biochem.* **59**, 1750–2 (1995).

8. Sarkar, M. & Schachter, H. Cloning and expression of Drosophila melanogaster UDP-GlcNAc:α-3-D-mannoside β1,2-N-acetylglucosaminyltransferase I. *Biol. Chem.* (2001). doi:10.1515/BC.2001.028

9. Zhang, W., Betel, D. & Schachter, H. Cloning and expression of a novel UDP-GlcNAc :α-D-mannoside β1,2-N- acetylglucosaminyltransferase homologous to UDP-GlcNAc :α-3-D-mannoside β1,2-N-acetylglucosaminyltransferase I. *Biochem. J* **361**, 153–162 (2002).

10. Dohi, K., Isoyama-Tanaka, J., Tokuda, T. & Fujiyama, K. Recombinant Expression and Characterization of N-Acetylglucosaminyltransferase I Derived from Nicotiana tabacum. *J. Biosci. Bioeng.* **109**, 388–391 (2010).

11. Strasser, R. *et al.* Molecular Cloning and Characterization of cDNA Coding for 1,2N-Acetylglucosaminyltransferase I (GlcNAc-TI) from Nicotiana tabacum. *Glycobiology* **9**, 779–785 (1999).

12. Hamilton, B. S. *et al.* A Library of Chemically Defined Human N-Glycans Synthesized from Microbial Oligosaccharide Precursors. *Sci. Rep.* **7**, (2017).

13. Oh-eda, M. *et al.* Overexpression of the Golgi-Localized Enzyme α-Mannosidase IIx in Chinese Hamster Ovary Cells Results in the Conversion of Hexamannosyl- N -Acetylchitobiose to Tetramannosyl- N -Acetylchitobiose in the N-Glycan-Processing Pathway. *Eur. J. Biochem.* **268**, 1280–1288 (2001).

14. Akama, T. O. & Fukuda, M. N. N-Glycan Structure Analysis Using Lectins and an α-Mannosidase Activity Assay. in 304–314 (2006). doi:10.1016/S0076-6879(06)16020-6

15. Strasser, R. *et al.* Molecular cloning and characterization of *Arabidopsis thaliana* Golgi α-mannosidase II, a key enzyme in the formation of complex N-glycans in plants. *Plant J.* **45**, 789–803 (2006).

16. Rose, D. R. *Structure, Mechanism and Inhibition of Golgi α-Mannosidase II*. *Curr. Opin. Struct. Biol.* **22**, 558–562 (2012).

17. Rabouille, C. *et al.* The Drosophila GMII gene encodes a Golgi alpha-mannosidase II. *J. Cell Sci.* **112 (Pt 1**, 3319–3330 (1999).

18. Li, J., Zhang, J., Lai, B., Zhao, Y. & Li, Q. Cloning, Expression, and Characterization of Capra hircus Golgi α-Mannosidase II. *Appl. Biochem. Biotechnol.* **177**, 1241–1251 (2015).

19. Paschinger, K. *et al.* A Deletion in the Golgi alpha-Mannosidase II Gene of Caenorhabditis elegans Results in Unexpected Non-Wild-Type N-Glycan Structures. *J. Biol. Chem.* **281**, 28265–77 (2006).

20. Moremen, K. W. & Robbins, P. W. Isolation, Characterization, and Expression of cDNAs Encoding Murine a-Mannosidase II, a Golgi Enzyme That Controls Conversion of High Mannose to Complex N-Glycans. *J. Cell Biol.* **115**, (1991).

21. Malissard, M. *et al.* Recombinant Soluble beta-1,4-Galactosyltransferases Expressed in Saccharomyces cerevisiae. Purification, Characterization and Comparison with Human Enzyme. *Eur. J. Biochem.* **239**, 340–348 (1996).

22. Nakazawa, K., Furukawa, K., Narimatsu, H. & Kobata, a. Kinetic study of human beta-1,4-galactosyltransferase expressed in E. coli. *J. Biochem.* **113**, 747–53 (1993).

23. Ito, T. *et al.* Highly Oriented Recombinant Glycosyltransferases: Site-specific Immobilization of Unstable Membrane Proteins by Using Staphylococcus aureus Sortase A. *Biochemistry* **49**, 2604–2614 (2010).

24. Palacpac, N. Q. *et al.* Stable Expression of Human β1,4-Galactosyltransferase in Plant Cells Modifies N-linked Glycosylation Patterns. *Proc. Natl. Acad. Sci.* **96**, 4692–4697 (1999).

25. Heinzler, R., Fischöder, T., Elling, L. & Franzreb, M. Toward Automated Enzymatic Glycan Synthesis in a Compartmented Flow Microreactor System. *Adv. Synth. Catal.* **361**, 4506–4516 (2019).

26. Nishiguchi, S. *et al.* Highly efficient oligosaccharide synthesis on water-soluble polymeric primers by recombinant glycosyltransferases immobilised on solid supports. *Chem. Commun. (Camb).* 1944–5 (2001). doi:10.1039/b104896c

27. Shibatani, S., Fujiyama, K., Nishiguchi, S., Seki, T. & Maekawa, Y. Production and Characterization of Active Soluble Human l,4-Galactosyltransferase in Escherichia coli as a Useful Catalyst in Synthesis of the Gal GlcNAc Linkage. *J. Biosci. Bioeng.* **91**, 85–87 (2001).

28. Hesselink, T. *et al.* Expression of natural human β1,4-GalT1 variants and of non-mammalian homologues in plants leads to differences in galactosylation of N-glycans. *Transgenic Res.* (2014). doi:10.1007/s11248-014-9806-z

29. Geisler, C., Mabashi-Asazuma, H., Kuo, C. W., Khoo, K. H. & Jarvis, D. L. Engineering β1,4-galactosyltransferase I to reduce secretion and enhance N-glycan elongation in insect cells. *J. Biotechnol.* (2015). doi:10.1016/j.jbiotec.2014.11.013

30. Park, J.-E., Lee, K.-Y., Do, S.-I. & Lee, S.-S. Expression and Characterization of β-1,4-Galactosyltransferase from Neisseria meningitidis and Neisseria gonorrhoeae. *BMB Rep.* **35**, 330–336 (2002).

The enzyme is alternatively involved in an O-linked glycosylation pathway. Examples of suitable enzymes involved in O-linked glycosylation pathways include core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1 (C1GalT1) and Alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase 1 (ST6GalNac1).

The immobilised and biotinylated enzyme that is involved in a glycosylation pathway may be produced by a method comprising the steps of:

(i) co-expressing a polynucleotide encoding a solubility tag, a catalytic domain of said enzyme, a linker and an AviTag with a polynucleotide encoding the enzyme BirA;
(ii) adding biotin; and

contacting the expressed polypeptide with streptavidin or avidin.

The first polynucleotide for use in the first aspect of the invention therefore encodes a solubility tag, a catalytic domain of an enzyme involved in a glycosylation pathway, a linker and an AviTag.

As described herein in relation to these polynucleotides, the components are listed in the order that they appear, from N-terminus to C-terminus, i.e with the sequence encoding a solubility tag at the N-terminus and the AviTag at the C-terminus.

By a "solubility tag" is meant a peptide, polypeptide or protein which, when fused to a polypeptide or protein of interest, causes the polypeptide or protein of interest to be soluble or enhances the solubility of the polypeptide or protein of interest. Solubility tags are a type of "fusion tag", which is the general term for a peptide, polypeptide or protein which, when fused to a polypeptide or protein of interest, imparts certain biochemical properties to the polypeptide or protein of interest. Fusion tags are generally added to a polypeptide or protein of interest at the genetic level by fusing the gene encoding the polypeptide or protein of interest to the gene encoding the fusion tag.

Any suitable solubility tag can be used, and will be known to a person skilled in the art. Suitable solubility fusion tags are listed in Table 1 below and are discussed further in Costa *et al.,* Frontiers in

Microbiology, Vol. 5, Article 63, pages 1-20 (2014), which is incorporated herein by reference in its entirety.

Table 4

List of possible solubility fusion tags

Protein	Advantages	Disadvantages
Glutathione s-transferase-GST	Protects against proteolysis and stabilises molecule. Suitable for one step purification.	Does not always enhance solubility Not suitable for proteins >100kDa or with charged residues
Maltose binding protein-MBP	Increases expression, folding and solubility.	Large size may be a load to the protein leading to instabilities and aggregation. Might require smaller tags i.e. His for higher purity
Small ubiquitin related modulators - SUMO	Increases stability of proteins Easy to remove Can be used for increased expression in mammalian, insects and yeast	Might need chaperone tags for increased stability Does not support purification
Mistic	Fusion tag for the purification of proteins	
Ecotin	Membrane associating protein Enhances expression of membrane proteins	Does not support purification

Solubility tags for use in the invention include MBP, GST, SUMO, mistic and ecotin.

The MBP may have the polynucleotide sequence of polynucleotides 1-1101 of any one of SEQ ID NOs: 1-3 as shown herein, or a polynucleotide sequence having at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity thereto.

The first polynucleotide for use in the first aspect of the invention encodes a catalytic domain of an enzyme involved in a glycosylation pathway, for example an N- or O-linked glycosylation pathway. It is not necessary for the polynucleotide to encode the full polypeptide sequence of the enzyme; a truncated version of the enzyme including the catalytic domain is sufficient. Typically, the truncated version of the enzyme excludes the transmembrane domain. However, in some embodiments, the polynucleotide encodes other domains of the enzyme in addition to the catalytic domain and in some embodiments the polypeptide encodes the full polypeptide sequence of the enzyme.

Catalytic domains of enzymes for use in the present invention can be readily identified using information available from publicly available databases (NCBI, etc).

The catalytic domain of an enzyme for use in the present invention may have the polynucleotide sequence of polynucleotides 1174-2424 of SEQ ID NO: 1 (NtGnTI) or polynucleotides 1174-2199 (or 1174-2200) of SEQ ID NO: 2 (hGnTI), or polynucleotides 1174-1983 of SEQ ID NO: 3 (hGalT), or a polynucleotide sequence having at least 85%, at least 86%, at least 87%, at least 88%, at least 89%,

at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to any of these sequences.

The first polynucleotide for use in the first aspect of the invention also encodes a linker. Linkers are well known in the art of preparing recombinant fusion proteins. The linker is typically chosen such that is does not interfere with the activity of the catalytic domain of the enzyme and/or with polypeptide or protein folding. Linker sequences are typically flexible, being made up primarily of amino acids such as glycine, alanine and serine, which do not have bulky side chains likely to restrict flexibility. Typically, the linker is a flexible linker encoding up to 10 amino acids, for example 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids and in particular 2, 4, 6, 8, or 10 amino acids. The linker sequence may be repeated to form a longer linker. Each linker may be formed from one, two, three or four repeats of a shorter linker sequence. Suitable linkers for use in the invention include glycine-serine (GS) linkers, optionally where the GS is repeated, e.g. GSGS (SEQ ID NO: 6), GSGSGS(SEQ ID NO: 7), GSGSGSGS (SEQ ID NO: 8) or GSGSGSGSGS (SEQ ID NO: 9), or having the sequence $(GGGGS)_n$(SEQ ID NO: 10), where n is typically 1, 2 or 3. Alternatively, rigid linkers may be desirable. Such linkers include glycine-glycine (GG), $(EAAAK)_n$ (SEQ ID NO: 11), where n is typically 1, 2 or 3, and $(XP)_n$, where X is any amino acid (preferably Ala, Lys or Glu) and n is typically 1, 2 or 3. Suitable fusion protein linkers are discussed in Chen *et al.,* Advanced Drug Delivery Reviews 65 (2014) 1357-1369, which is incorporated herein by reference in its entirety.

The linker may have the polynucleotide sequence of polynucleotides 2431-2436 of SEQ ID NO: 1, polynucleotides 2206-2211 of SEQ ID NO: 2 or 1990-1995 of SEQ ID NO: 3 as shown herein (all of which are the same), or a polynucleotide sequence having at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to any of these sequences.

The first polynucleotide for use in the first aspect of the invention also encodes an AviTag. AviTag is also known as the Acceptor Peptide, AP. AviTag enables the enzymatic labelling (using *E. coli* biotin ligase, BirA) of a protein of interest by biotin, which binds to streptavidin or avidin. The binding between biotin and streptavidin or avidin is one of the strongest known non-covalent biological interactions.

Several peptide sequences have been described for BirA-mediated biotinylation. In the present invention, the AviTag amino acid sequence is typically GLNDIFEAQKIEWHE (SEQ ID NO: 12). Alternatively, the AviTag consensus amino acid sequence may be LXZIFEAQ**K**IEWR (SEQ ID NO: 13), where X = any amino acid and Z = any amino acid except for L, V, I, W, F or Y. Other suitable amino acid sequences include BioTag (ALNDIFEAQ**K**IEWHA (SEQ ID NO: 14)), BLRP (Biotin ligase recognition peptide) which contains a core of AviTag and is 23 residues long (MAGGLNDIFEAQ**K**IEWHEDTGGS (SEQ ID NO: 15)) and BirA Substrate Peptide (BSP), LHHILDAQ**K**MVWNHR (SEQ ID NO: 16). For the purposes of this invention, these sequences all fall within the definition of "AviTag".

The AviTag may be encoded by the polynucleotide sequence of polynucleotides 2437-2481 of SEQ ID NO: 1, polynucleotides 2212-2256 of SEQ ID NO: 2 or 1996-2040 of SEQ ID NO: 3 as shown herein, or a polynucleotide sequence having at least 85%, at least 86%, at least 87%, at least 88%, at least 89%,

at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to any of these sequences.

Enzymatic biotinylation with *E. coli* biotin ligase (BirA) is highly specific in covalently attaching biotin to the 15 amino acid AviTag peptide, giving a homogeneous product with high yield. This is discussed in detail by Fairhead and Howarth (Site-specific biotinylation of purified proteins using BirA; Methods Mol Biol. 2015; 1266: 171–184.), which is incorporated herein by reference in its entirety. Fairhead and Howarth describe procedures for AviTag insertion by inverse PCR, purification of BirA fused to glutathione-S-transferase (GST-BirA) from *E. coli*, BirA biotinylation of purified protein, and gel-shift analysis by SDS-PAGE to quantify the extent of biotinylation. Further discussion of biotinylation, including enzymatic biotinylation using BirA can be found in Kay et al. (Methods Mol Biol. 2009; 498: 185-196).

Vectors containing N- or C-terminal AviTag sequences are also commercially available (e.g. from Avidity or from Genecopoeia). These are suitable for bacterial, mammalian or cell-free expression; some plasmids have BirA downstream for coexpression. Suitable vectors can be found here:

https://www.addgene.org/search/catalog/plasmids/?q=Avitag&page_size=20&expression=Mammalian+Expression

Typically, co-expression takes place in a bacterial host cell. Typically, the bacterial host cell is an *E. coli* cell.

The streptavidin or avidin is typically coated on a support, for example a bead.

The first polynucleotide for use in the first aspect of the invention encodes a solubility tag, a catalytic domain of an enzyme involved in a glycosylation pathway, a linker and an AviTag. The polynucleotide sequences of the constructs used in the Examples are as follows:

Abbreviations used are: hGnTI: human GnTI; NtGnTI: *Nicotiana tabacum* GnTI; hGalT: human GalT

MBP-NtGnTI-linker

MBP: 1-1101

NtGnTI (underlined): 1174-2424

Linker: 2431-2436

AviTag: 2437-2481

ATGAAAATCGAAGAAGGTAAACTGGTAATCTGGATTAACGGCGATAAAGGCTATAACGGTCTCGCTGAAGTC
GGTAAGAAATTCGAGAAAGATACCGGAATTAAAGTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATTC
CCACAGGTTGCGGCAACTGGCGATGGCCCTGACATTATCTTCTGGGCACACGACCGCTTTGGTGGCTACGCTC
AATCTGGCCTGTTGGCTGAAATCACCCCGGACAAAGCGTTCCAGGACAAGCTGTATCCGTTTACCTGGGATGC
CGTACGTTACAACGGCAAGCTGATTGCTTACCCGATCGCTGTTGAAGCGTTATCGCTGATTTATAACAAAGATC
TGCTGCCGAACCCGCCAAAAACCTGGGAAGAGATCCCGGCGCTGGATAAAGAACTGAAAGCGAAAGGTAAG
AGCGCGCTGATGTTCAACCTGCAAGAACCGTACTTCACCTGGCCGCTGATTGCTGCTGACGGGGGTTATGCGT

TCAAGTATGAAAACGGCAAGTACGACATTAAAGACGTGGGCGTGGATAACGCTGGCGCGAAAGCGGGTCTG
ACCTTCCTGGTTGACCTGATTAAAAACAAACACATGAATGCAGACACCGATTACTCCATCGCAGAAGCTGCCTT
TAATAAAGGCGAAACAGCGATGACCATCAACGGCCCGTGGGCATGGTCCAACATCGACACCAGCAAAGTGAA
TTATGGTGTAACGGTACTGCCGACCTTCAAGGGTCAACCATCCAAACCGTTCGTTGGCGTGCTGAGCGCAGGT
ATTAACGCCGCCAGTCCGAACAAAGAGCTGGCAAAAGAGTTCCTCGAAAACTATCTGCTGACTGATGAAGGT
CTGGAAGCGGTTAATAAAGACAAACCGCTGGGTGCCGTAGCGCTGAAGTCTTACGAGGAAGAGTTGGTGAA
AGATCCGCGTATTGCCGCCACTATGGAAAACGCCCAGAAAGGTGAAATCATGCCGAACATCCCGCAGATGTC
CGCTTTCTGGTATGCCGTGCGTACTGCGGTGATCAACGCCGCCAGCGGTCGTCAGACTGTCGATGAAGCCCTG
AAAGACGCGCAGACTAATTCGAGCTCGAACAACAACAACAATAACAATAACAACAACCTCGGGATCGAGGGA
AGGATTTCACATATGGCGACGCAATCGGAATATGCGGATCGCTTGGCAGCGGCGATTGAAGCCGAAAACCAT
TGCACGTCACAGACCCGCTTACTGATCGATCAAATCAGTCAGCAACAAGGACGCATTGTAGCCCTGGAGGAAC
AGATGAAACGTCAGGATCAGGAGTGTCGCCAATTACGTGCTTTGGTTCAGGATCTTGAGTCGAAAGGGATTA
AGAAATTGATCGGCAATGTTCAAATGCCTGTTGCTGCAGTAGTGGTCATGGCCTGCAATCGTGCGGATTACCT
CGAGAAAACCATCAAATCCATCCTGAAGTATCAGATTAGCGTTGCACCGAAATACCCTCTGTTTATCTCCCAAG
ATGGTTCTCATCCGGATGTCCGCAAACTGGCGTTAAGCTACGATCAACTGACCTATATGCAGCATCTGGATTTT
GAACCGGTGCACACTGAACGTCCTGGCGAATTAATCGCGTATTACAAAATTGCACGCCACTACAAATGGGCCC
TTGACCAGCTCTTTTACAAGCACAACTTTAGCCGGGTGATCATTCTTGAGGACGATATGGAAATTGCCCCAGAC
TTCTTCGACTTCTTTGAAGCCGGAGCTACTCTGCTGGATCGCGATAAGTCGATTATGGCGATCAGTAGCTGGA
ACGATAACGGGCAGATGCAGTTTGTGCAAGATCCCTATGCTTTATATCGCTCAGACTTCTTTCCGGGTCTGGGT
TGGATGTTGAGTAAATCGACATGGGACGAACTGAGCCCGAAATGGCCGAAAGCTTACTGGGATGACTGGTTG
CGCCTGAAGGAAAACCATCGTGGTCGTCAGTTCATTCGCCCGGAAGTGTGTCGTAGCTATAACTTTGGTGAAC
ATGGTAGCAGTCTGGGCCAGTTCTTTAAACAGTATCTGGAACCCATCAAACTCAATGACGTCCAGGTCGACTG
GAAATCCATGGATCTTTCTTATCTGCTGGAGGACAATTACGTGAAACACTTTGGCGATCTGGTGAAGAAAGCG
AAACCGATTCATGGTGCCGACGCAGTGCTGAAAGCGTTTAACATTGATGGGGATGTTCGCATTCAGTACCGTG
ATCAGCTGGACTTTGAAGATATTGCACGTCAGTTTGGCATTTTCGAAGAGTGGAAAGATGGCGTACCACGTGC
GGCCTATAAAGGCATCGTAGTGTTCCGCTATCAGACGTCACGCCGGGTTTTCCTCGTCGGCCCAGACTCTCTGC
AGCAACTGGGCAATGAAGATACCGAATTCGGTTCT

MBP-hGnTI-linker

MBP: 1-1101

hGnTI (underlined): 1174-2199

Linker: 2206-2211

AviTag: 2212-2256

ATGAAAATCGAAGAAGGTAAACTGGTAATCTGGATTAACGGCGATAAAGGCTATAACGGTCTCGCTGAAGTC
GGTAAGAAATTCGAGAAAGATACCGGAATTAAAGTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATTC
CCACAGGTTGCGGCAACTGGCGATGGCCCTGACATTATCTTCTGGGCACACGACCGCTTTGGTGGCTACGCTC
AATCTGGCCTGTTGGCTGAAATCACCCCGGACAAAGCGTTCCAGGACAAGCTGTATCCGTTTACCTGGGATGC
CGTACGTTACAACGGCAAGCTGATTGCTTACCCGATCGCTGTTGAAGCGTTATCGCTGATTTATAACAAAGATC
TGCTGCCGAACCCGCCAAAAACCTGGGAAGAGATCCCGGCGCTGGATAAAGAACTGAAAGCGAAAGGTAAG
AGCGCGCTGATGTTCAACCTGCAAGAACCGTACTTCACCTGGCCGCTGATTGCTGCTGACGGGGGTTATGCGT
TCAAGTATGAAAACGGCAAGTACGACATTAAAGACGTGGGCGTGGATAACGCTGGCGCGAAAGCGGGTCTG

ACCTTCCTGGTTGACCTGATTAAAAACAAACACATGAATGCAGACACCGATTACTCCATCGCAGAAGCTGCCTT
TAATAAAGGCGAAACAGCGATGACCATCAACGGCCCGTGGGCATGGTCCAACATCGACACCAGCAAAGTGAA
TTATGGTGTAACGGTACTGCCGACCTTCAAGGGTCAACCATCCAAACCGTTCGTTGGCGTGCTGAGCGCAGGT
ATTAACGCCGCCAGTCCGAACAAAGAGCTGGCAAAAGAGTTCCTCGAAAACTATCTGCTGACTGATGAAGGT
CTGGAAGCGGTTAATAAAGACAAACCGCTGGGTGCCGTAGCGCTGAAGTCTTACGAGGAAGAGTTGGTGAA
AGATCCGCGTATTGCCGCCACTATGGAAAACGCCCAGAAAGGTGAAATCATGCCGAACATCCCGCAGATGTC
CGCTTTCTGGTATGCCGTGCGTACTGCGGTGATCAACGCCGCCAGCGGTCGTCAGACTGTCGATGAAGCCCTG
AAAGACGCGCAGACTAATTCGAGCTCGAACAACAACAACAATAACAATAACAACAACCTCGGGATCGAGGGA
AGGATTTCACAtatgGCGGTGATTCCGATCCTGGTCATTGCGTGTGACCGTTCGACCGTGCGTCGTTGCCTGGA
TAAACTGTTGCATTACCGCCCGTCTGCCGAGCTGTTTCCAATCATTGTTTCTCAAGACTGCGGCCATGAGGAAA
CCGCTCAAGCGATCGCAAGCTATGGTAGCGCGGTTACGCACATCCGCCAGCCGGATCTGTCCAGCATCGCGGT
TCCGCCGGATCACCGCAAATTCCAAGGTTACTACAAAATTGCGCGTCATTATCGTTGGGCGCTGGGTCAGGTA
TTTCGCCAGTTTCGCTTTCCGGCAGCGGTCGTCGTCGAGGATGATCTGGAGGTTGCCCCAGACTTCTTCGAGT
ACTTCCGTGCGACGTATCCGTTGCTGAAGGCAGATCCGTCCCTGTGGTGCGTCAGCGCGTGGAATGATAACG
GTAAAGAGCAGATGGTGGATGCCAGCCGTCCTGAACTGCTGTACCGTACCGACTTCTTTCCGGGCCTGGGTTG
GCTGCTGTTGGCTGAACTGTGGGCGGAACTGGAGCCGAAGTGGCCGAAAGCATTTTGGGACGATTGGATGC
GTCGCCCGGAACAGCGCCAGGGCCGTGCCTGTATTCGCCCGGAGATTAGCCGCACCATGACGTTTGGTCGCA
AGGGCGTGAGCCACGGCCAGTTCTTTGACCAGCATCTGAAATTCATTAAGCTGAATCAGCAATTCGTTCACTTC
ACCCAACTGGACCTGAGCTACTTGCAACGTGAGGCGTATGATCGTGACTTCTTGGCGCGTGTCTATGGTGCTC
CGCAACTGCAAGTCGAGAAAGTGCGCACGAACGATCGTAAGGAGCTGGGTGAGGTGCGCGTGCAGTACACC
GGCCGTGACAGCTTTAAGGCCTTCGCCAAGGCGCTGGGCGTCATGGACGACCTGAAAAGCGGCGTTCCTCGT
GCGGGTTATCGTGGTATTGTGACCTTTCAGTTCCGTGGTCGTCGCGTTCATCTGGCACCGCCGCTGACCTGGG
AAGGCTACGACCCGAGCTGGAACgaattCGGTTCT

MBP-hGalT-linker

MBP: 1-1101

hGalT (underlined): 1174-1983

Linker: 1990-1995

AviTag: 1996-2040

ATGAAAATCGAAGAAGGTAAACTGGTAATCTGGATTAACGGCGATAAAGGCTATAACGGTCTCGCTGAAGTC
GGTAAGAAATTCGAGAAAGATACCGGAATTAAAGTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATTC
CCACAGGTTGCGGCAACTGGCGATGGCCCTGACATTATCTTCTGGGCACACGACCGCTTTGGTGGCTACGCTC
AATCTGGCCTGTTGGCTGAAATCACCCCGGACAAAGCGTTCCAGGACAAGCTGTATCCGTTTACCTGGGATGC
CGTACGTTACAACGGCAAGCTGATTGCTTACCCGATCGCTGTTGAAGCGTTATCGCTGATTTATAACAAAGATC
TGCTGCCGAACCCGCCAAAAACCTGGGAAGAGATCCCGGCGCTGGATAAAGAACTGAAAGCGAAAGGTAAG
AGCGCGCTGATGTTCAACCTGCAAGAACCGTACTTCACCTGGCCGCTGATTGCTGCTGACGGGGGTTATGCGT
TCAAGTATGAAAACGGCAAGTACGACATTAAAGACGTGGGCGTGGATAACGCTGGCGCGAAAGCGGGTCTG
ACCTTCCTGGTTGACCTGATTAAAAACAAACACATGAATGCAGACACCGATTACTCCATCGCAGAAGCTGCCTT
TAATAAAGGCGAAACAGCGATGACCATCAACGGCCCGTGGGCATGGTCCAACATCGACACCAGCAAAGTGAA
TTATGGTGTAACGGTACTGCCGACCTTCAAGGGTCAACCATCCAAACCGTTCGTTGGCGTGCTGAGCGCAGGT
ATTAACGCCGCCAGTCCGAACAAAGAGCTGGCAAAAGAGTTCCTCGAAAACTATCTGCTGACTGATGAAGGT

CTGGAAGCGGTTAATAAAGACAAACCGCTGGGTGCCGTAGCGCTGAAGTCTTACGAGGAAGAGTTGGTGAA
AGATCCGCGTATTGCCGCCACTATGGAAAACGCCCAGAAAGGTGAAATCATGCCGAACATCCCGCAGATGTC
CGCTTTCTGGTATGCCGTGCGTACTGCGGTGATCAACGCCGCCAGCGGTCGTCAGACTGTCGATGAAGCCCTG
AAAGACGCGCAGACTAATTCGAGCTCGAACAACAACAACAATAACAATAACAACAACCTCGGGATCGAGGGA
AGGATTTCACAtatgGCCTGCCCTGAGGAAAGCCCACTGTTGGTGGGCCCAATGCTGATCGAGTTTAACATGCC
GGTGGACCTGGAACTGGTGGCGAAACAGAACCCGAACGTCAAAATGGGCGGCCGTTACGCACCGCGTGACT
GCGTTAGCCCGCACAAAGTCGCGATCATTATTCCGTTCCGCAATCGCCAAGAGCATCTGAAGTACTGGCTGTA
CTATCTGCATCCAGTTCTGCAACGTCAGCAATTGGACTACGGTATTTACGTTATCAATCAAGCCGGCGACACGA
TCTTTAATCGTGCTAAGTTGCTGAATGTTGGTTTTCAAGAAGCGCTGAAAGACTACGACTACACCTGTTTCGTG
TTCTCCGACGTTGACCTGATTCCGATGAATGATCACAATGCGTACCGCTGTTTTTCTCAGCCGCGTCACATCAG
CGTAGCGATGGATAAGTTTGGTTTCAGCCTGCCGTATGTGCAGTATTTTGGTGGCGTCAGCGCACTGAGCAAG
CAACAGTTTCTCACGATTAACGGTTTCCCGAACAACTATTGGGGTTGGGGTGGCGAAGATGATGATATCTTCA
ACCGTCTGGTGTTCCGTGGTATGAGCATTAGCCGCCCGAACGCTGTGGTTGGCCGTTGCCGTATGATTCGTCA
TAGCCGCGACAAGAAAAATGAACCGAATCCTCAGCGTTTCGATCGTATCGCACACACCAAAGAAACTATGTTG
AGCGACGGCTTAAACAGCCTGACCTATCAAGTCTTGGATGTTCAACGCTATCCGCTGTACACGCAGATTACCG
TGGACATTGGCACCCCGAGCGaattCGGTTCT

The first polynucleotide for use in the first aspect of the invention may therefore comprise the polynucleotide sequence of any one of SEQ ID NOs: 1-3, or a polynucleotide sequence having at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the polynucleotide sequence of any one of SEQ ID NOs: 1-3. Preferably said sequence identity is at least 90% or at least 95%.

Sequence identity may be assessed by any convenient method. However, for determining the degree of sequence identity between sequences, computer programmes that make pairwise or multiple alignments of sequences are useful, for instance EMBOSS Needle or EMBOSS stretcher (both Rice, P. et al., Trends Genet., 16, (6) pp276-277, 2000) may be used for pairwise sequence alignments while Clustal Omega (Sievers F et al., Mol. Syst. Biol. 7:539, 2011) or MUSCLE (Edgar, R.C., Nucleic Acids Res. 32(5):1792-1797, 2004) may be used for multiple sequence alignments, though any other appropriate programme may be used. Whether the alignment is pairwise or multiple, it must be performed globally (i.e. across the entirety of the reference sequence) rather than locally.

Sequence alignments and % identity calculations may be determined using for instance standard Clustal Omega parameters: matrix Gonnet, gap opening penalty 6, gap extension penalty 1. Alternatively, the standard EMBOSS Needle parameters may be used: matrix BLOSUM62, gap opening penalty 10, gap extension penalty 0.5. Any other suitable parameters may alternatively be used.

The immobilised and biotinylated enzyme for use in the first aspect of the invention is typically produced by the method described above. However, the immobilised and biotinylated enzyme does not necessarily need to be produced by enzyme engineering. Alternatively, the immobilised enzyme may be biotinylated using chemical means. An example of carrying out biotinylation using chemical means is given in the Examples herein in relation to DmManII. Methods for chemical biotinylation

are well known in the art (as described for example in Kay et al. (Methods Mol Biol. 2009; 498: 185-196).

Typically, the glycoprotein is derived from any cellular or cell-free expression system that can perform glycosylation.

Typically, the glycoprotein is a therapeutic or prophylactic glycoprotein, for example a hormone, enzyme, antibody or fragment thereof (for example an Fc fragment, Fab (antigen binding) fragment, Fv (variable) fragment or an scFv) or vaccine.

In a second aspect, the present invention provides a polynucleotide encoding a solubility tag, a catalytic domain of an enzyme involved in a glycosylation pathway, a linker and an AviTag. This corresponds to the first polynucleotide as defined in relation to the first aspect of the invention. The features of the polynucleotide of the second aspect of the invention are as described above in relation to the first aspect of the invention.

In a third aspect, the present invention provides a vector comprising the polynucleotide as defined in relation to the first aspect of the invention, or a polynucleotide of the second aspect of the invention.

In a fourth aspect, the present invention provides a host cell comprising the vector of the third aspect of the invention. Typically, the host cell is a bacterial host cell. Typically, the host cell is an *E. coli* host cell.

In a fifth aspect, the present invention provides a polypeptide encoded by the polynucleotide as defined in relation to the first aspect of the invention, or a polynucleotide of the second aspect pf the invention.

In a sixth aspect, the present invention provides an *in vitro* method of producing an immobilised and biotinylated enzyme that is involved in a glycosylation pathway, comprising the steps of:

(i) co-expressing a polynucleotide as defined in relation to the first aspect of the invention or a polynucleotide of the second aspect of the invention with a polynucleotide encoding the enzyme BirA;
(ii) adding biotin; and
(iii) contacting the expressed polypeptide with streptavidin or avidin.

Typically, both the poynucleotide as defined in relation to the first aspect of the invention or polynucleotide of the second aspect of the invention are included in a vector, such as a plasmid, for co-expression. Typically, co-expression takes place in a bacterial host cell. Typically, the bacterial host cell is an *E. coli* cell. Alternatively, the host cell may be a eukaryotic cell. The skilled person will be able to choose a vector for co-expression that is suitable for use in the host cell being used, for example a eukaryotic vector for co-expression in a eukaryotic host cell.

The streptavidin or avidin is typically coated on a support, for example a bead.

The method of the sixth aspect of the invention is referred to herein as a one step immobilisation/purification process. The method may include additional steps, for example as shown in Figure 2, which is a schematic showing the one step immobilisation/purification process of

engineered enzymes. Enzymes are typically produced in *E.coli* cells. The cells are then broken (lysed). A desalting step may be included to remove biotin that could bind on the streptavidin beads instead of the biotinylated enzymes. After desalting, the solution is mixed with the beads and only the biotinylated enzyme is captured. With a simple centrifugation step the immobilised enzyme (enzyme bound on beads) is recovered and the unbound material is discarded. This method for one-step immobilisation/purification of the engineered enzymes from the crude extract eliminates the need for any chromatography steps whilst lowering processing time and cost.

The main challenge in the manufacture of therapeutic glycoproteins is that the reactions to add the sugars are not templated, but rather rely on the availability of the sugar donors and the (promiscuous) enzymes that perform the addition reaction, leading to a heterogeneous product. Industry generally tackles the heterogeneity problem in one of two ways, alone or in combination. (1) glycoengineering, or the genetic modification of the host cells to alter the expression level of the proteins involved in sugar addition or (2) changes in process conditions such as the composition and rate of feed addition to alter the metabolism and modify the resulting glycans. Both strategies are slow and laborious due to the complicated mapping between bioprocess conditions, glycosyltransferase expression, metabolism, and final glycoform. Additionally, it is not possible to glycoengineer the host cells once the product has gained regulatory approval.

The aim of the present invention is to provide an alternative system (referred to herein as an Artificial Golgi) to modify glycoproteins using immobilised enzymes (glycosyltransferases and glycosidases). Different glycosylation-enabling enzymes can be biotinylated and immobilised on streptavidin coated supports. Immobilised enzymes are then used to modify oligosaccharide structures on glycoproteins. After each step of modification, the beads with enzymes can be efficiently removed, and no residual enzyme/beads will interfere with the next round of immobilised enzyme and glycosylation modification. The benefits from this are as follows: a) enzyme promiscuity is addressed b) enzymes can be reused for the same process or even a different one thus lowering the cost, c) It is a protein-independent system meaning it can be applied on any therapeutic glycoprotein of interest. This way an array of therapeutic drugs (e.g. hormones, enzymes, antibodies, vaccines etc) carrying homogeneous glycosylation profiles can be produced and d) the system is modular allowing to create different oligosaccharide structures on-demand by simply changing the order or nature of enzymes. This way, the Artificial Golgi has the potential to reconstruct a whole human N-linked glycosylation pathway in proteins that are produced in non-mammalian hosts as well as create novel structures with applications in vaccine development.

The present invention provides a novel system for changing the oligosaccharide structures on proteins. The system comprises a set of immobilized glycosyltransferase and glycosidase enzymes (for example GnTI, ManII and GalT) and it can be used to alter the oligosaccharide structure of a glycoprotein after harvest from a cell-based expression. The inventors have developed a method to engineer and *in vivo* modify the target glycosyltransferase enzymes to enable their production in a bacterial host and their subsequent immobilisation (Figure 1c & d). Following immobilisation, the enzymes are reacted separately with the desired oligosaccharide structures or glycoprotein (Figure 1b). The immobilised enzymes can be easily recovered and reused, which facilitates product purification and the process economics. The inventors have demonstrated that the enzymes can be reused up to 7 times without loss of activity. The Artificial Golgi described herein is widely applicable in the biopharmaceutical industry given the importance of glycosylation on therapeutic

protein function, stability and efficacy of medicinal proteins. It can also be used for the construction of glycoconjugate vaccines.

The Artificial Golgi reactor relies on the use of immobilised enzymes. Naturally, these enzymes compete against the same substrate leading to the production of multiple undesired structures (Figure 1a). The use of immobilised enzymes allows them to be easily removed from solution once their reaction is completed (thus preventing any undesired cross-reactivity whilst eliminating the need for intermediate purifications) and then add the next enzyme of the reaction scheme (Figure 1b). This way different enzymes never come into contact thus the invention addresses enzyme competition whilst ensuring product homogeneity.

The plug-and-play nature of the system and its potential as a platform to produce human-like therapeutics deriving from non-mammalian cell-based systems (or indeed any system for glycosylation) are other advantages of the system.

As an *in vitro* strategy that would be applied post-production, the Artificial Golgi should enable significant improvements in the speed and cost of bioprocess development for the production of new therapeutics. It would allow manufacturers to use a basic platform process for the production of all of their products, with the ability to tune the glycoform post-manufacture from a variety of host cells including cheaper yeast production systems. Other host systems include mammalian cells, insects, plants etc as well as cell-free systems such as glycoengineered bacteria cell-free systems, mammalian cell-free etc.

As described herein, O-linked glycosylation, the post-translational addition of glycans to Serine/Theronine, contributes to various physiological process such as immunity and development. O-linked glycans serve as biomarkers for blood type identification as well as cancer development, while they serve as target for glycan binding proteins. O-linked glycosylation is less complex than N-linked glycosylation. However, the lack of templated reactions, the enzyme promiscuity and enzyme availability can lead to multiple glycan structures and consequently heterogeneity. An immobilised enzyme cascade can in principle address this leading to the production of the desired glycan structure in homogeneity (Figure 1e. and f.). Such a system can further facilitate efforts to produce bespoke vaccine targets, biomarkers and glycan epitopes.

Preferred features for the second and subsequent aspects of the invention are as described for the first aspect *mutatis mutandis*.

The present invention will now be described with reference to the following Examples, which are present for the purposes of illustration only. In the Examples, reference is made to a number of Figures in which:

Figure 1: **a.** N-linked glycosylation pathway of GnTI, ManII and GalT, where enzyme promiscuity naturally exists. GalT recognises multiple structures as substrates leading to an array of possible products; **b**. Reaction cascade with immobilised enzymes to address GalT promiscuity of pathway shown in **a**; **c**. Enzyme engineering for *in vivo* biotinylation. The catalytic domain of the target enzyme is fused to a Maltose Binding Protein (MBP) in the N-Terminus and AviTag in the C-terminus. To ensure functionality, a small two-residue Glycine-Serine (GS) linker was inserted before the AviTag. The biotin ligase BirA recognises AviTag and can perform enzymatic biotinylation; **d.** Immobilisation of biotinylated enzyme on streptavidin coated supports; **e.** and **f.**: mucin-type O-

linked glycosylation. e. Example pathway where heterogeneity naturally exists; f. application of Artificial Golgi Reactor to achieve homogeneity

Figure 2: One-step immobilisation/purification of engineered enzymes. Enzymes are produced in *E.coli* cells. The cells are broken and the soluble content including the enzymes are extracted via centrifugation. A desalting step is necessary to remove biotin that could bind on the streptavidin beads instead of the biotinylated enzymes. After desalting, the solution is mixed with the beads and only the biotinylated enzyme is captured. With a simple centrifugation step the immobilised enzyme (enzyme bound on beads) is recovered and the unbound material is discarded.

Figure 3 *In vivo* biotinylation confirmation using a gel shift assay. Each lane was loaded with and without BirA and StV in the absence of reducing agent. a. Confirmation of biotinylation for MBP-hGnTI-AviTag; b. Confirmation of biotinylation for MBP-NtGnTI-AviTag; c. Confirmation of biotinylation for MBP-hGalT-AviTag; StV: streptavidin, Avi: AviTag; hGnTI: human GnTI; NtGnTI: *Nicotiana tabacum* GnTI; hGalT: human GalT

Figure 4 shows immobilisation of enzymes on StV beads a. Immobilisation of NtGnTI; b. Immobilisation of hGalT; C. immobilisation of DmManII*; d. Immobilisation of hGnTI

*: DmManII was biotinylated using commercially available chemical reagents, as described in the Example

Figure 5 shows confirmation of activity of immobilised NtGnTI, as monitored by MALDI-TOF MS. a. 0h, starting material acceptor sugar M5 b. overnight reaction, product GM5

Figure 6 shows confirmation of activity of immobilised DmManII as monitored by MALDI-TOF MS. a. 0h, starting sugar GM5 b. Overnight reaction, product GM3.

Figure 7 shows confirmation of activity of immobilised hGalT as monitored by MALDI-TOF. Starting material is GlcNAc cannot be detected as a single sugar hence no 0h reaction spectra)

Figure 8 shows sequential reaction of immobilised NtGnTI-DmManII-hGalT as monitored by MALDI-TOF MS. Each step was performed overnight. a. Starting substrate M5; b. Conversion of M5 to GM5 by immobilised NtGnTI; c. Conversion of GM5 to GM3 by immobilised DmManII. Reaction approached completion; d. Conversion of GM3 to GalGM3 by immobilised hGalT. Reaction approached completion.

Figure 9 shows the use of immobilised hGalT to enhance galactosylation of antibodies. CE electropherograms for humanised IgG produced in CHO cells (chIgG) treatment with immobilised on magnetic StV beads hGalT.: a. untreated chIgG; b. chIgG and hGalT.

Figure 10 shows CE electropherograms for IgG from human serum (hIgG) treatment with immobilised on magnetic StV beads hGalT.:a. untreated hIgG; b. hIgG and hGalT.

Figure 11 shows CE electropherograms for IgG from rabbit serum (rIgG) treatment with immobilised on magnetic StV beads hGalT.:a. untreated rIgG; b. rIgG and hGalT.

EXAMPLES

Generation of MBP-Enzyme-Avitag constructs

Restriction digestion was used to construct all plasmids, and enzymes used were purchased by NEB®. The MBP-Linker-AviTag vector was generated first and used as a basis for all glycosyltransferases.

The sequence of GS linker-AviTag was chemically synthesised as single stranded DNA and ligated into double stranded. The annealed oligonucleotides encode the AviTag peptide sequence GLNDIFEAQKIEWHE, carry a GS linker (N-terminus) and recognition sites for *EcoRI* (N-terminus) and *HindIII* (C-terminus).

Name	Sequence
AviTag forward	AATTCGGTTCTGGTCTTAATGATATTTTTGAAGCTCAGAAGATTGAATGGCATGAAA (SEQ ID NO: 4)
AviTag reverse	AGCTTTTCATGCCATTCAATCTTCTGAGCTTCAAAAATATCATTAAGACCAGAACCG (SEQ ID NO: 5)

To generate the MPB-AviTag vector, AviTag was cloned in a PMAL-C5X vector (MBP-encoding vector, NEB) using restriction digestion.

All glycosyltransferase enzymes were cloned into the MBP-Avitag vector using restriction digestion to generate MBP-Glycosyltransferase-linker-AviTag

pMAL-c5X-GS linker-AviTag	Derivative of pMAL-c5X with GS linker-AviTag generated using *EcoRI* and *HindIII.*
pMAL-c5X-GT-AviTag	Derivative of pMAL-c5X-GSlinker-AviTag with glycosyltransferase generated using *NdeI* and *EcoRI*.

Enzyme expression and *in vivo* biotinylation using BirA

All glycosyltransferases (NtGnTI, hGnTI, hGalT) were expressed as MBP-fusion proteins in *E. coli* Origami 2 DE3. To support *in vivo* biotinylation, all cells were co-transformed with the plasmid encoding for the glycosyltransferase (MBP-Enzyme-GS linker-AviTag) and pBirAcm (the plasmid encoding for BirA).

- Growth: *E. coli* was transformed with the plasmid encoding for the GT of interest and encoding for BirA was inoculated into LB media with the appropriate antibiotic marker and incubated at 37˚C with shaking overnight (~16 hours).
- Expression: When Optical density ($OD600_{nm}$) was 0.6-0.8, expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM and incubated overnight at 20°C. d-biotin (20µM final concentration) was also supplemented to increase biotinylation yield.

Chemical biotinylation of DmManII

DmManII was kindly provided by Dr. David Rose and Dr. Doug Kuntz (University of Waterloo, Ontario Canada) in 10mM Tris-HCl pH 7.5 and 100mM NaCl. DmManII was subsequently diluted to 1mg/ml.

The Lightning-Link® Rapid Biotin Type B Labelling Kit - 3 x up to 200µg Ab, from Expedeon, Ltd, was used for the chemical biotinylation of DmManII. Steps followed were as described by the manufacturer:

Equilibrate all materials and prepared reagents to room temperature prior to use. Add 1 µL of Modifier reagent to each 10 µL of antibody to be labeled and mix gently. Remove cap from vial of Biotin Conjugation Mix and pipette the antibody sample (with added Modifier reagent) directly onto the lyophilized material. Resuspend gently by withdrawing and re-dispensing the liquid once or twice using a pipette. Replace cap on the vial and leave standing for 15 minutes in the dark at room temperature (20-25°C). Longer incubation times, such as overnight, have no negative effect on the conjugation. After incubating for 15 minutes (or more), add 1 µL of Quencher reagent for every 10 µL of antibody used and mix gently. The conjugate can be used after 5 minutes. The conjugates do not require purification

One step-immobilisation/purification

Figure 2 is a schematic showing the one step immobilisation/purification process.

- Lysis by sonication

To prevent protein degradation all lysis steps were performed on ice. *E. coli* cell pellets were thawed and resuspended in an appropriate volume (~5 ml for every gram of cells) of storage buffer (20mM Tris-HCl pH7.4, 200 mM NaCl and 5% glycerol) supplemented with 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 1mg/mL lysozyme from chicken egg (Sigma-Aldrich). Note, that in the case of hGnTI, NaCl concentration in storage buffer was 500mM. To lyse the cells the samples were sonicated for 5 min, 30% amplitude and 10 sec on/off pulses (Fisherbrand). The lysate was centrifuged (12000 *xg*, 30 min, 4 ^{o}C) to remove the lysed bacteria using rotor.

- Desalting

To prevent protein degradation all desalting steps were performed at 4^{o}C. Desalting of the soluble fraction (SF) was necessary to remove any unreacted d-biotin from the SF that could hinder immobilisation

Desalting of SF was performed using PD-10 desalting columns (GE Healthcare) and by following the protocol described by the manufacturer. Briefly, columns were equilibrated 5 times in storage buffer (20mM Tris-HCl pH7.4, 200 mM NaCl and 5% glycerol, except for hGnTI where 500mM NaCl was used), then 2.5 mL of SF were loaded and once completely entered the column, 3.5 mL of storage buffer was added. The flow-through was collected, aliquoted, flash-frozen (dry ice and EtOH bath) and stored at -80^{o}C until the one-step purification/ immobilisation.

- One-step immobilisation/purification

For enzyme immobilisation, Streptavidin silica particles 1% w/v. 1.0-1.4 µM from Spherotech or the Dynabeads C1, streptavidin coated magnetic beads, 1% w/v from ThermoFisher, were used. The storage buffer of the beads was removed either by centrifugation (5min, 5000 xg for silica beads) or by the use of a magnet. The particles were subsequently washed 3 times with 0.1M Tris-HCl pH 7.4 by resuspending and centrifuging / magnet separation.

The immobilisation for silica particles was performed as follows: desalted SF was mixed with the washed and pelleted beads, prepared as described earlier. Here, a volumetric ratio of 1:2 (SF: StV beads) was used e.g. 25µL of SF to 50µL of StV beads (volume corresponds to volume of particles before washing and pelleting). For DmManII, since the concentration was known (1mg/mL) an appropriate volume based on the binding capacity of beads, as specified by the manufacturer, was used. The samples were then diluted with 0.1M Tris-HCl, pH 7.4 at a final immobilisation volume of 1mL and incubated in a rotary shaker for 1 hour at 4°C. To scale up experiments, larger volumes of SF and StV beads were used, while keeping the 1:2 ratio. Note, that the immobilisation volume was always 1mL (make up with 0.1M Tris). Following immobilisation, the samples were centrifuged for 10min at 3000 xg, the supernatant was removed, and the pelleted beads were resuspended in storage buffer (20mM Tris pH 7.4, 200mM NaCl and 5% glycerol). The centrifugation steps were repeated, and the washing process was performed 3 times in total. At the end of the washes, the immobilised enzyme was used in the glycosylation reactions described.

Sequential glycosylation reactions on artificial glycans

The activity assay of immobilised NtGnTI consisted of 0.5µM M5 glycan, 2.5mM UDP-GlcNAc, 100mM MES pH 6.5 and 1mM $MnCl_2$, at 25°C overnight on a shaking platform (note: tube was placed vertically). The immobilisation experiment was scaled up by a factor of 4 (i.e. 100µL SF and 200µL silica StV beads) After the end of the reactions, the immobilised enzyme was removed by centrifugation (5min, 5000xg). The supernatant was aliquoted and used for MALDI-TOF MS analysis and as a substrate for the reaction of DmManII.

The activity assay of immobilised DmManII consisted of 0.1mM $ZnSO_4$, 50mM MES, pH 5.6 and 0.4mM substrate from immobilised NtGnTI's reaction. Reaction was performed overnight at 37°C on a shaking platform (note: tube was placed vertically). After the end of the reactions, the immobilised enzyme was removed by centrifugation (5min, 5000xg). The supernatant was aliquoted and used for MALDI-TOF MS analysis and as a substrate for the reaction of DmManII.

The activity assay of immobilised hGalT consisted of 6mM UDP-Gal, 80mM Tris-HCl pH 9, 10mM $MnCL_2$ and 0.3mM substrate from immobilised DmManII's reaction. Where described, 1µl of the alkaline phosphatase FastAp (Thermofischer scientific) was added to remove inhibitory products. Immobilisation experiment was scaled by a factor of 4 (i.e. 100µL SF and 200µL silica StV beads). Reaction was performed overnight at 37°C on a shaking platform (note: tube was placed vertically). After the end of the reactions, the immobilised enzyme was removed by centrifugation (5min, 5000xg). The supernatant was aliquoted and used for MALDI-TOF MS analysis.

Use of immobilised hGalT to alter galactosylation of full length IgGs

hGalT was immobilised on either silica StV beads (100µL SF and 200µL silica StV beads) or magnetic StV beads (i.e. 100µL SF and 40µL silica StV beads).

The antibodies used were a) purified humanised IgG produced in CHO cells (chIgG) b) IgG from human serum (Sigma-Aldrich, resuspended in deionised H2O and stored at 4°C); c) IgG from rabbit (Sigma-Aldrich, resuspended in deionised H2O and stored at 4°C)

The reaction consisted of the following components: 110µg IgG (chIgG / hIgG / rIgG), 20mM $MnCl_2$, 20mM Tris-HCl pH 7.5, 6mM UDP-Gal. The reaction took place overnight at 37°C on a shaking platform (note: tube was placed vertically). After the end of the reactions, the immobilised enzyme was removed by centrifugation (5min, 5000xg). The supernatant was used for CE analysis.

Table 5 - Collective results for galactosylation of IgGs using immobilised hGalT

	IgGs					
	chIgG		hIgG		rIgG	
hGalT / Glycoform	-	+	-	+	-	+
G0	ND	ND	13.4	10.7	29.0	8.2
G1	ND	ND	ND	ND	10.4	12.0
G1'	ND	ND	3.4	1.2	19.6	18.5
G2	5.6	5.3	3.6	3.0	6.9	27.1
G0F	54.2	3.7	16.1	5.8	5.1	ND
G1F	25.9	34.8	24.1	28.6	4.1	3.2
G1'F	9.9	8.2	16.3	16.9	7.7	5.5
G2F	4.4	48.0	23.2	33.8	4.0	13.9
Overall galactosylation	27.9	74.6	48.6	60.1	31.8	60.6
Terminal galactosylation	45.8	96.3	70.5	83.5	52.7	80.2

Reusability of immobilised hGalT and chIgG (humanised IgG produced in CHO cells)

Following completion of the overnight reaction of immobilised hGalT and chIgG, the immobilised enzyme was recovered by centrifugation (5min, 5000xg). The beads were resuspended in protein storage buffer (20mM Tris-HCl pH 7.5, 200mM NaCl and 5% glycerol) and recovered by centrifugation (5min, 5000xg). The washing step was performed 3 times in total. Finally, recovered beads were used for a new reaction as described in earlier. At the end of each reaction and after removing the immobilised enzymes, the supernatant containing chIgG was removed and used for CE analysis

Table 6 - Demonstration of reusability of immobilised hGalT: hGalT was reacted with chIgG

GalT treatment / Glycoforms	untreated chIgG	1st cycle	2nd cycle	3rd cycle	4th cycle	5th cycle	6th cycle	7th cycle
G0F	47.9	2.6	7.7	11.0	16.4	13.1	24.2	29.6
G1F	31.2	39.7	35.4	38.3	43.0	38.9	40.4	38.4
G1'F	11.9	7.9	10.2	11.4	12.8	13.6	12.8	12.5
G2	2.8	2.9	4.6	3.8	3.3	3.9	3.6	4.7
G2F	6.1	47.1	42.1	35.5	24.6	30.5	18.9	14.8
Overall galactosylation	30.5	73.7	69.5	64.1	55.7	60.6	49.2	45.0
Terminal Galactosylation	52.1	97.4	92.3	89.0	83.6	86.9	75.8	70.4

CLAIMS

1. An *in vitro* method of modifying the glycosylation pattern of a glycoprotein, comprising contacting said glycoprotein or a glycan component of said glycoprotein with an immobilised and biotinylated enzyme that is involved in a glycosylation pathway.

2. The method according to claim 1, wherein said glycoprotein or said glycan component is sequentially contacted with more than one immobilised and biotinylated enzyme that is involved in a glycosylation pathway.

3. The method according to claim 1 or 2, wherein the glycosylation pathway is an N- or O-linked glycosylation pathway.

4. The method according to claim 3, wherein the enzyme is eukaryotic.

5. The method according to claim 4, wherein the enzyme is β-1,4 galactosyltransferase (GalT), *N*-acetylglucosaminyltransferase I (GnTI), *N*-acetylglucosaminyltransferase II (GnTII), *N*-acetylglucosaminyltransferase III (GnTIII), *N*-acetylglucosaminyltransferase IV (GnTIV), *N*-acetylglucosaminyltransferase V (GnTV), sialyltransferase (SiaT), glycoprotein 6-alpha-L-fucosyltransferase (FucT) or α-mannosidase II (ManII).

6. The method according to claim 5, wherein the enzyme is GalT, GnTI or ManII.

7. The method according to any one of the preceding claims, wherein said immobilised and biotinylated enzyme is produced by a method comprising the steps of:

   (i) co-expressing a polynucleotide encoding a solubility tag, a catalytic domain of said enzyme, a linker and an AviTag with a polynucleotide encoding the enzyme BirA;

   (ii) adding biotin; and

   (iii) contacting the expressed polypeptide with streptavidin or avidin.

8. The method according to claim 7, wherein the solubility tag is selected from the group consisting of MBP, GST, SUMO, mistic and ecotin.

9. The method according to claim 7 or 8, wherein the linker is a flexible linker encoding up to 10 amino acids.

10. The method according to claim 9, wherein the linker encodes an amino acid sequence including GS, wherein the GS is optionally repeated.

11. The method according to any one of claims 7 to 10, wherein the AviTag amino acid sequence is GLNDIFEAQKIEWHE or wherein the AviTag consensus amino acid sequence is LXZIFEAQ**K**IEWR, where X = any amino acid and Z = any amino acid except for L, V, I, W, F or Y.

12. The method according to any one of the preceding claims, wherein said glycoprotein is a therapeutic or prophylactic glycoprotein.

13. The method according to claim 12, wherein said therapeutic or prophylactic glycoprotein is an antibody or fragment thereof, vaccine, hormone or enzyme.

14. A polynucleotide encoding a solubility tag, a catalytic domain of an enzyme that is involved in a glycosylation pathway, a linker and an AviTag, as defined in any one of claims 7 to 11.

15. A vector comprising the polynucleotide of claim 14.

16. A host cell comprising the vector of claim 15.

17. The host cell according to claim 16, which is a bacterial host cell.

18. The host cell according to claim 17, which is an *E. coli* host cell.

19. A polypeptide encoded by the polynucleotide of claim 14.

20. An *in vitro* method of producing an immobilised and biotinylated enzyme that is involved in a glycosylation pathway, comprising the steps of:

    (i) co-expressing a polynucleotide encoding a solubility tag, a catalytic domain of said enzyme, a linker and an AviTag, as defined in any one of claims 7 to 11, with a polynucleotide encoding the enzyme BirA;

    (ii) adding biotin; and

    (iii) contacting the expressed polypeptide with streptavidin or avidin.

21. The method according to claim 20, wherein co-expression takes place in a bacterial host cell.

22. The method according to claim 21, wherein the bacterial host cell is an *E. coli* cell.

23. The method according to any one of claims 20 to 22, wherein the streptavidin or avidin is coated on a support.

24. The method according to claim 23, wherein the support is a bead.

Figure 1

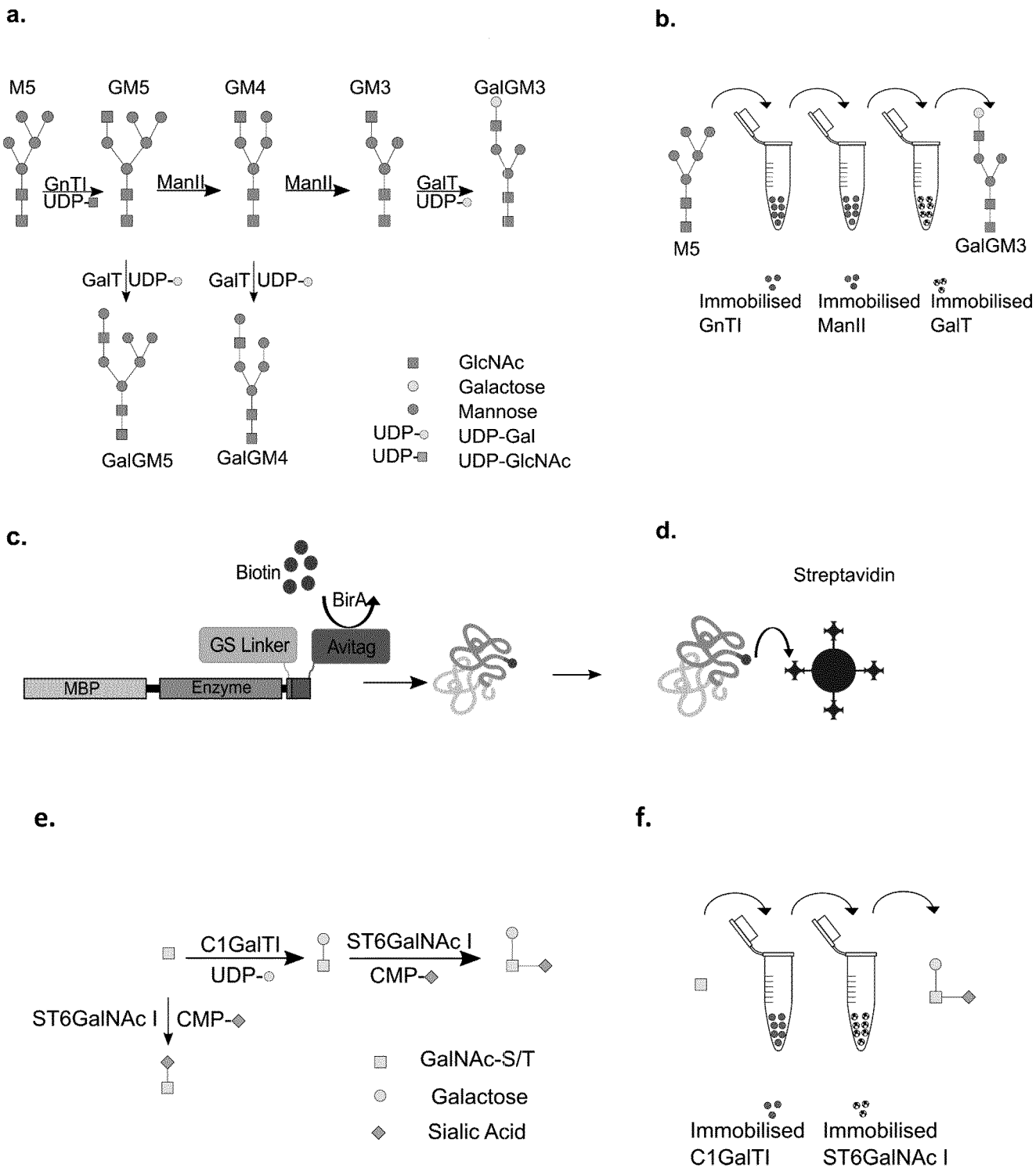

a.
M5
GM5
GM4
GM3
GalGM3
GnTI
UDP-
ManII
ManII
GalT
UDP-
GalT
UDP-
GalT
UDP-
GalGM5
GalGM4
GlcNAc
Galactose
Mannose
UDP-Gal
UDP-GlcNAc
b.
M5
GalGM3
Immobilised GnTI
Immobilised ManII
Immobilised GalT
c.
Biotin
BirA
GS Linker
Avitag
MBP
Enzyme
d.
Streptavidin
e.
C1GalTI
UDP-
ST6GalNAc I
CMP-
ST6GalNAc I
CMP-
GalNAc-S/T
Galactose
Sialic Acid
f.
Immobilised C1GalTI
Immobilised ST6GalNAc I

Figure 2

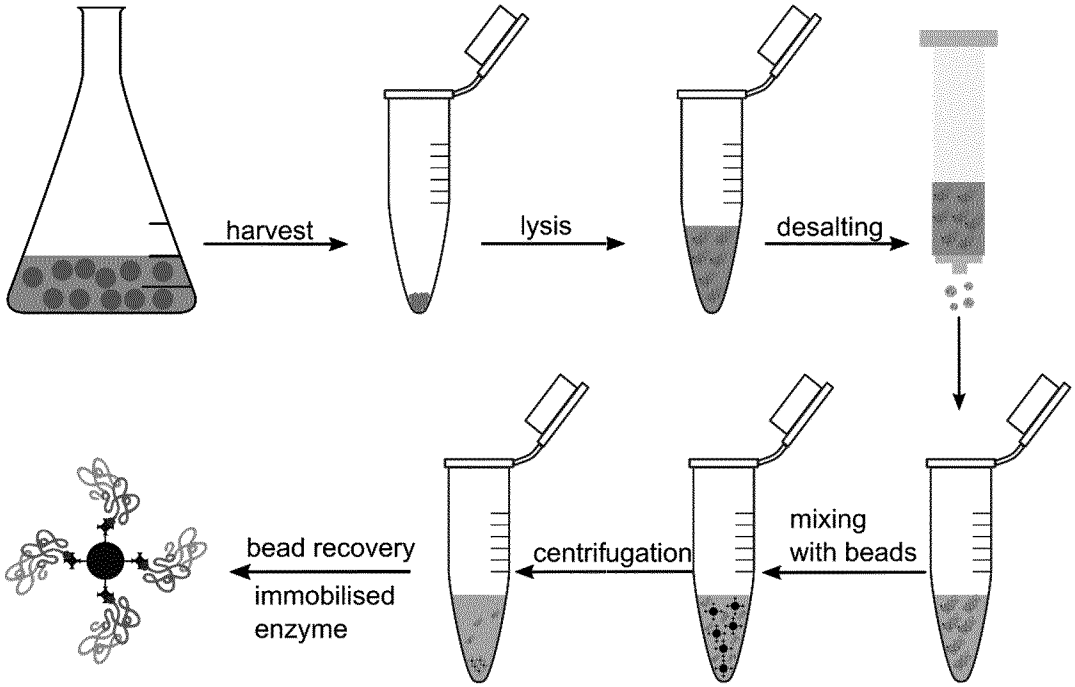


Figure 3

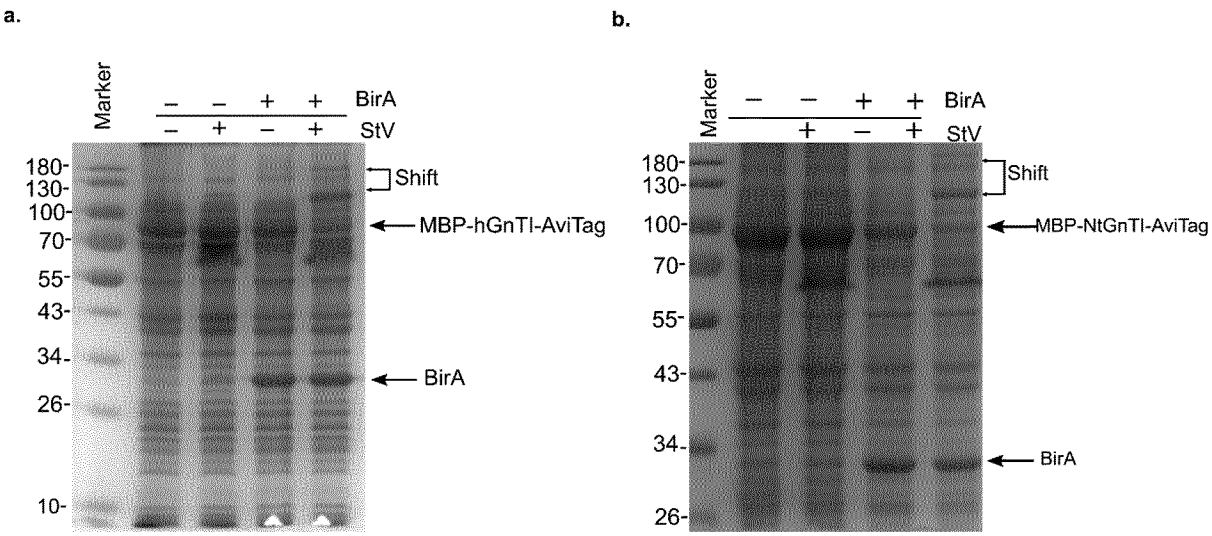
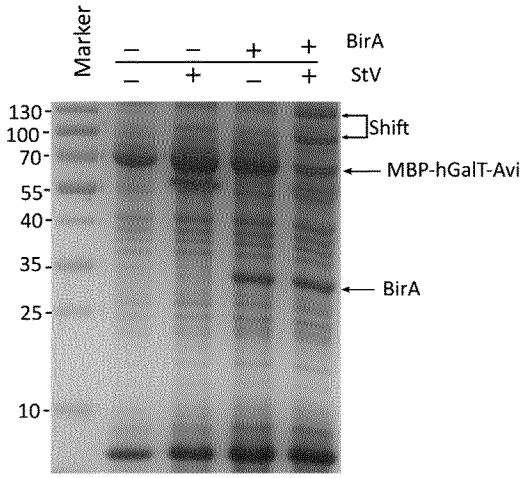
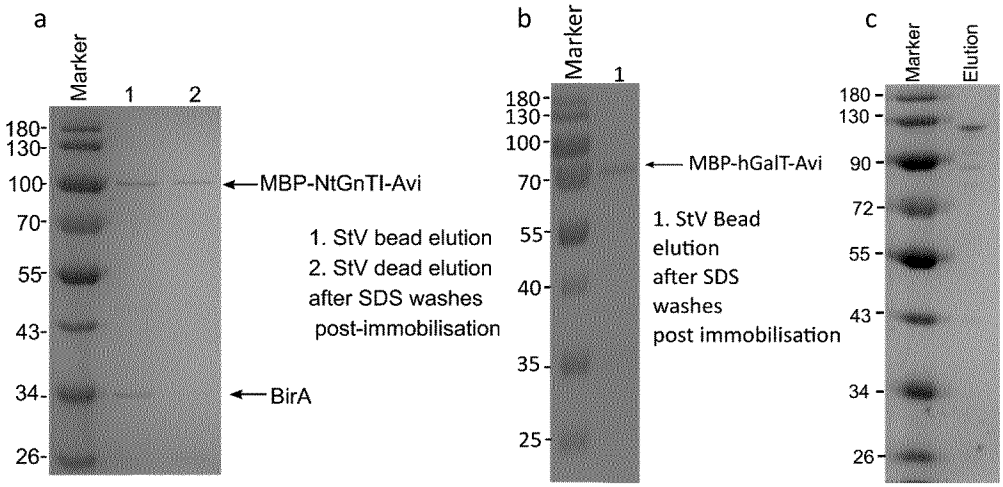


Figure 3



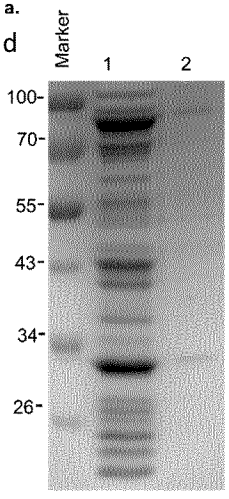
c.

Figure 4



a.

d



1. MBP-hGnTI-AviTag

Figure 5

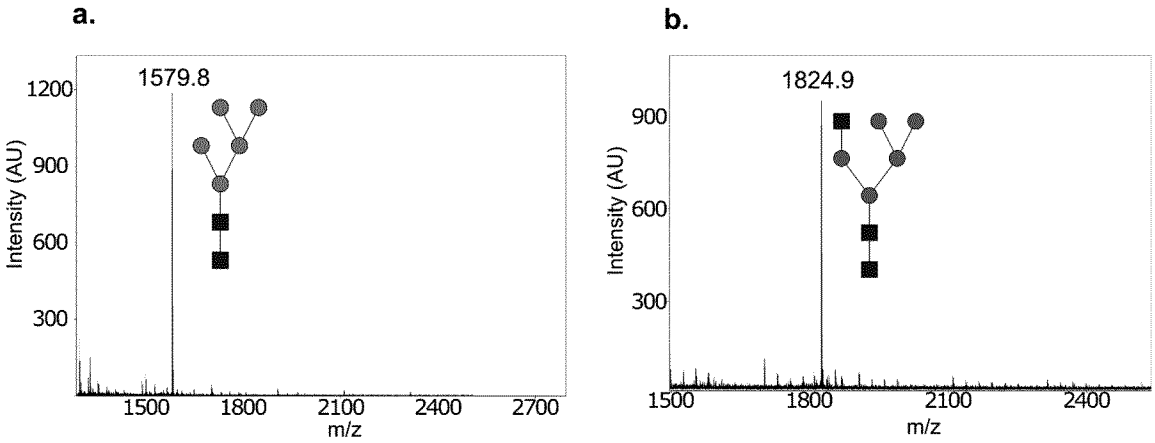


Figure 6

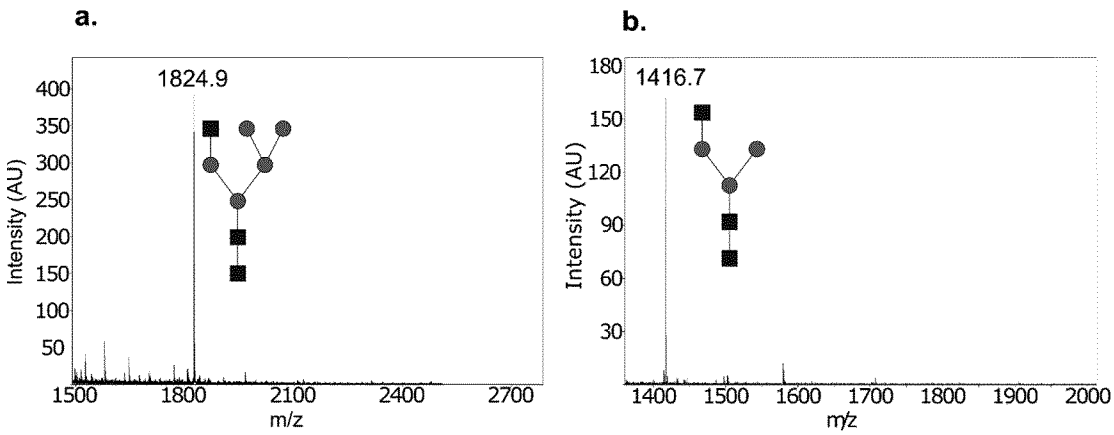


Figure 7

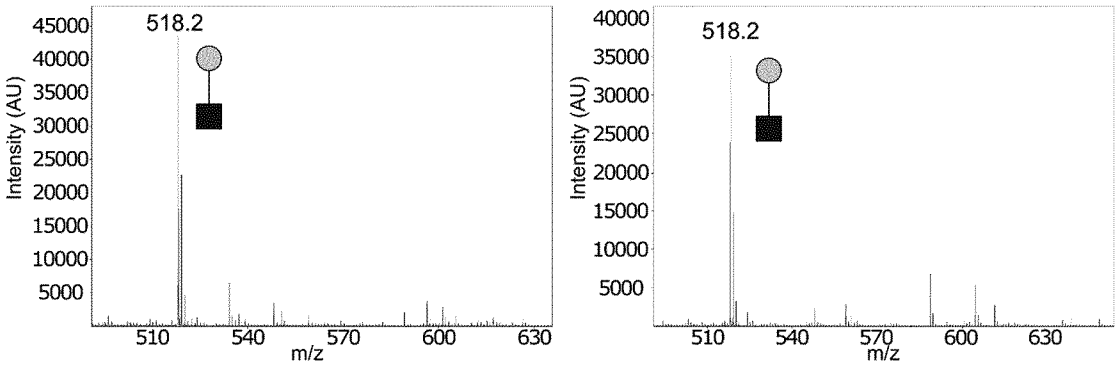
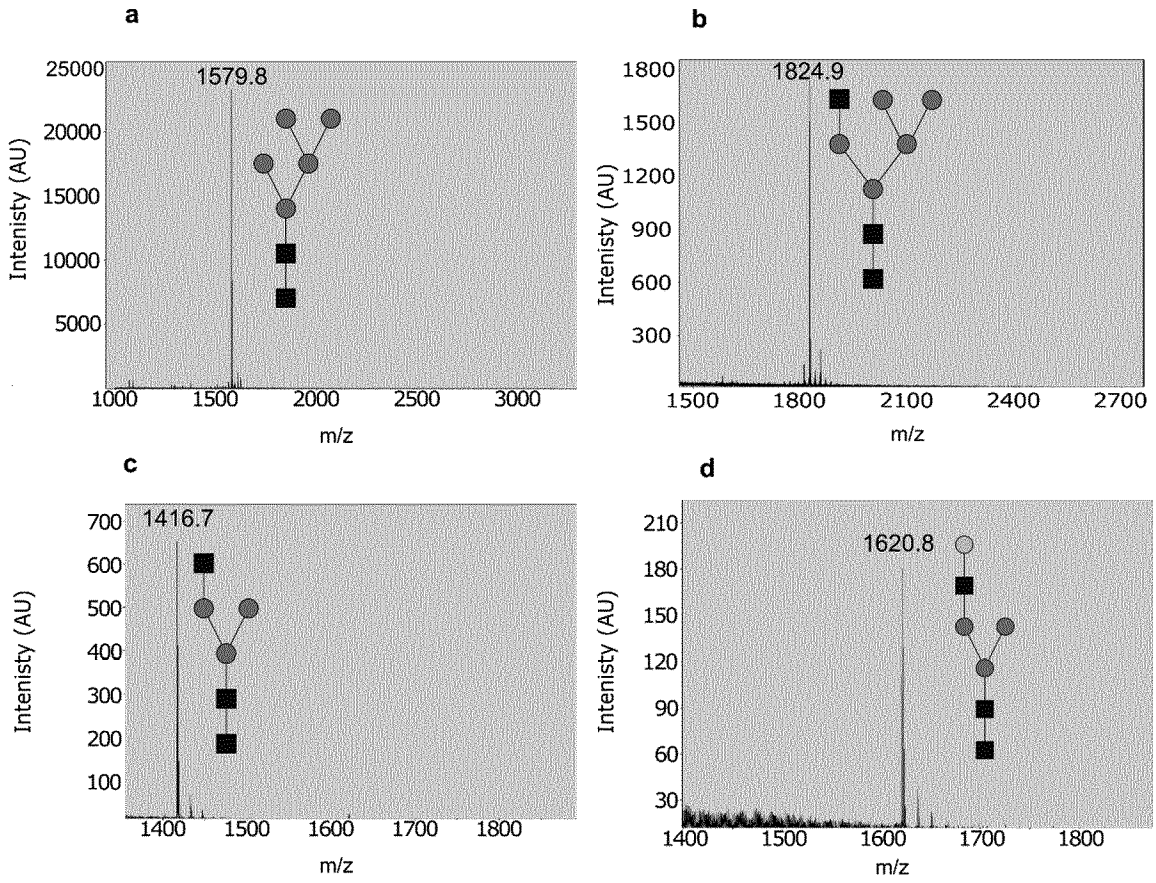


Figure 8


a
1579.8
Intenisty (AU)
m/z
b
1824.9
Intenisty (AU)
m/z
c
1416.7
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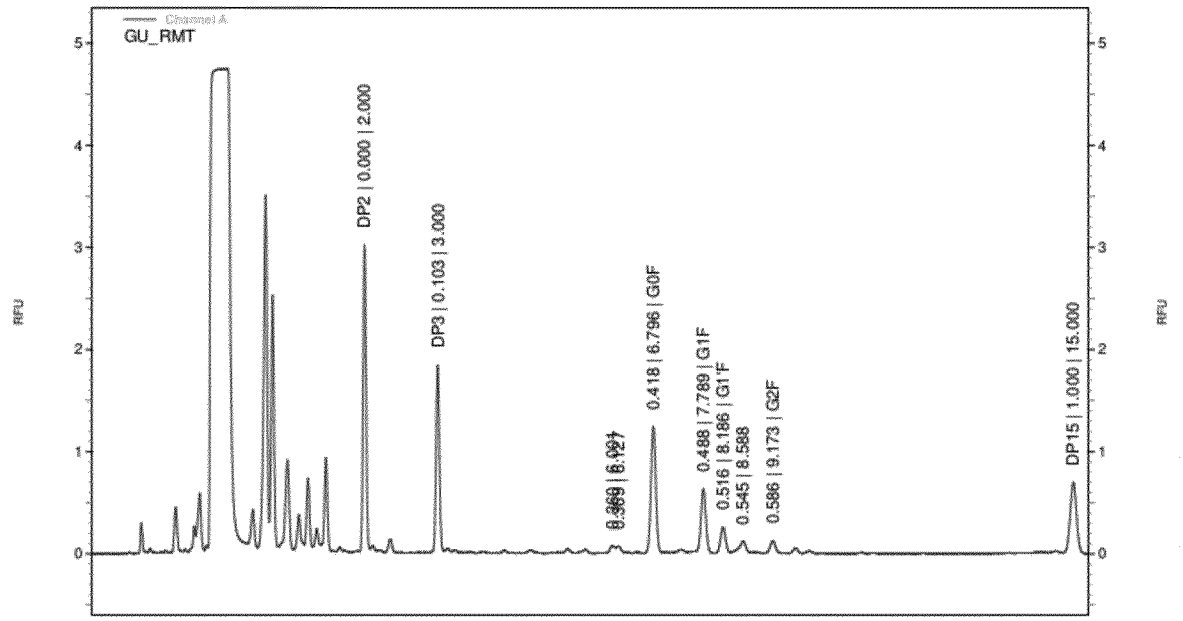
m/z
d
1620.8
Intenisty (AU)
m/z

Figure 9

a.

Electropherogram trace:



b.

Electropherogram trace:

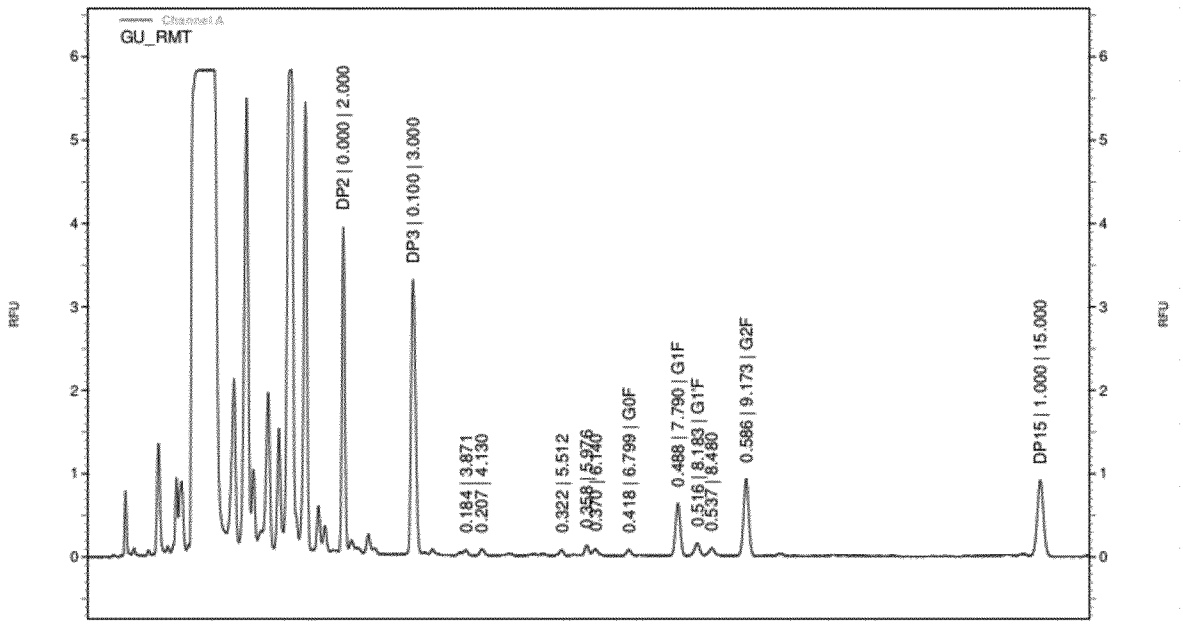
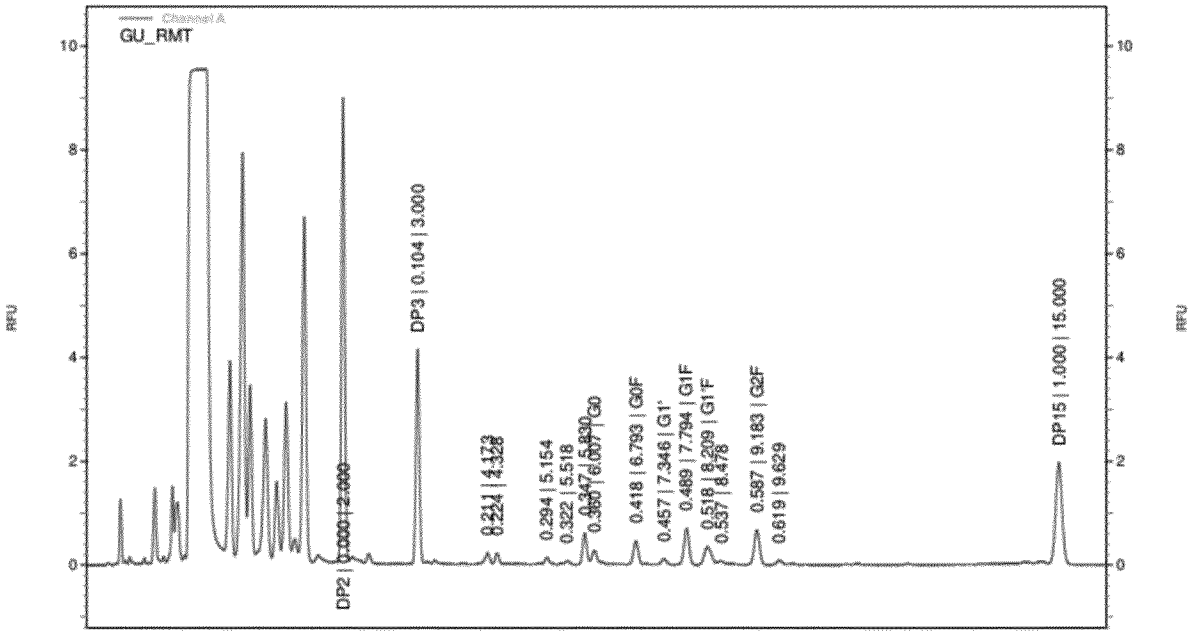


Figure 10

a.

Electropherogram trace:



b.

Electropherogram trace:

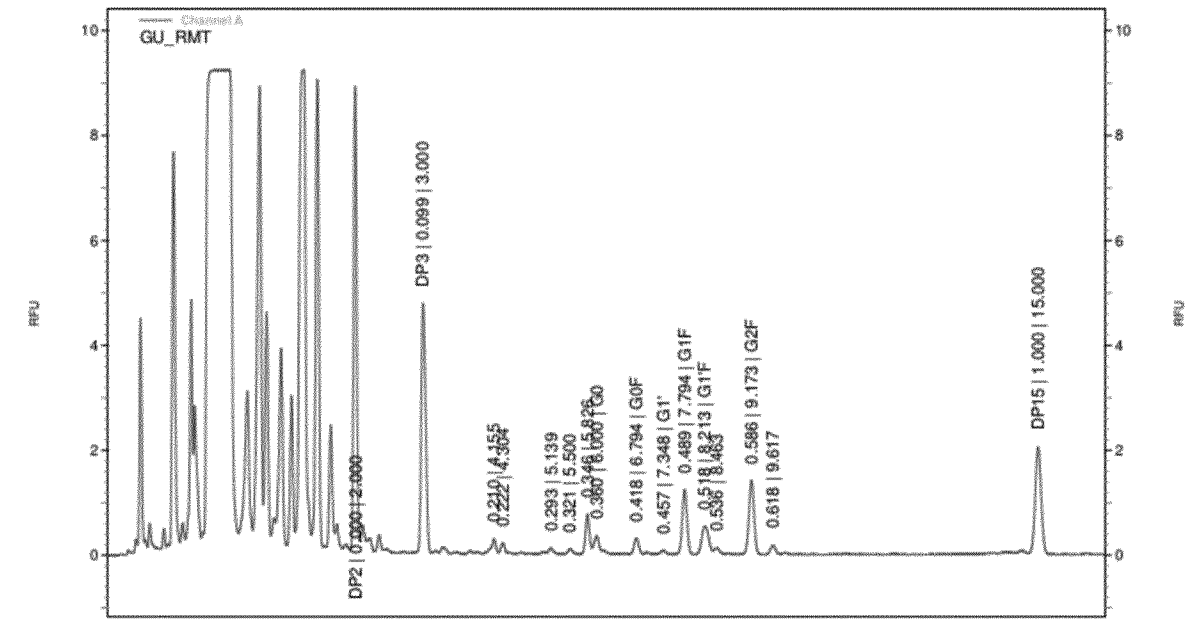
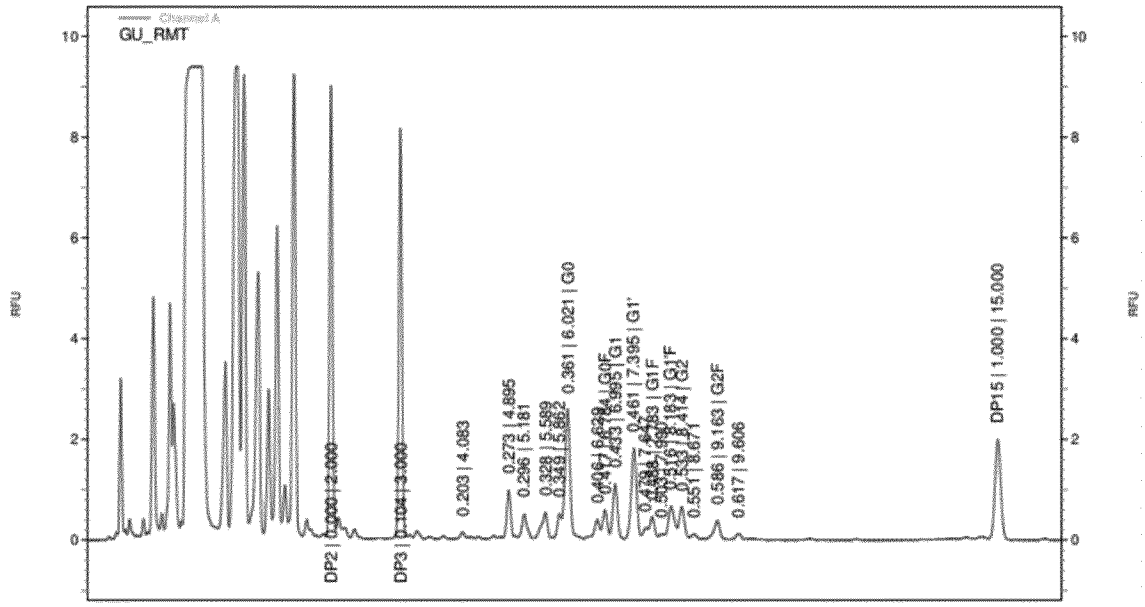


Figure 11

a.

Electropherogram trace:



b.

Electropherogram trace: