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(71) Applicant: **OBI PHARMA, INC.**; 19F., No. 3, Yuanqu St., Nangang Dist., Taipei City, 115 (TW).

(72) Inventor; and

(71) Applicant: **YU, Cheng-Der, Tony** [US/US]; c/o Obi Pharma, Inc., 6020 Cornerstone Ct., W, San Diego, CA 92121-3730 (US).

(72) Inventors: **HSIEH, Yih, Huang**; c/o OBI Pharma, Inc., 19F., No. 3, Yuanqu St., Nangang Dist., Taipei City, 115 (TW). **HSIEH, Yin-Cheng**; c/o OBI Pharma, Inc., 19F., No. 3, Yuanqu St., Nangang Dist., Taipei City, 115 (TW). **HUANG, Teng-Yi**; c/o OBI Pharma, Inc., 19F., No. 3,

Yuanqu St., Nangang Dist., Taipei City, 115 (TW). **TSAI, Yi-Chien**; c/o OBI Pharma, Inc., 19F., No. 3, Yuanqu St., Nangang Dist., Taipei City, 115 (TW). **WANG, Nan-Hsuan**; c/o OBI Pharma, Inc., 19F., No. 3, Yuanqu St., Nangang Dist., Taipei City, 115 (TW). **HU, Pu-Ling**; c/o OBI Pharma, Inc., 19F., No. 3, Yuanqu St., Nangang Dist., Taipei City, 115 (TW).

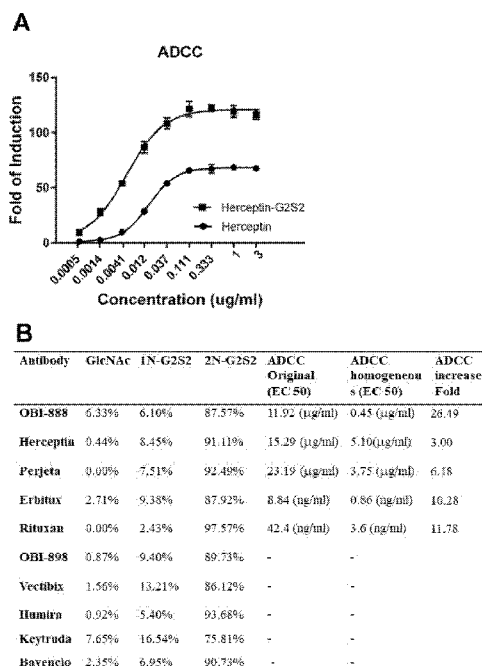
(74) Agent: **NORTON, Vicki, G.**; Duane Morris LLP, 750 B. Street, Suite 2900, San Diego, CA 92101-4681 (US).

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(54) Title: GLYCOSYNTASE VARIANTS FOR GLYCOPROTEIN ENGINEERING AND METHODS OF USE

Figure 7



(57) Abstract: The present disclosure relates to novel glycosynthase enzymes for glycoprotein engineering and/or homogeneous antibody remodeling. The enzyme variants, termed EndoSd-D232M and EndoSz-D234M, contain the glycan conjugation and/or modification activity at the conserved N297 glycosylation site of Fc region of an exemplary antibody. It has been demonstrated that the glycosynthase activities of EndoSd-D232M and EndoSz-D234M can be applied to various mAbs targeting different receptors, including, but not limited to, Globo H, S SEA-4, S SEA-3 series of receptors (OBI-888; Globo H ganglioside), Herceptin (Her 2 receptor), Perjeta (Her 2 receptor) and Vectibix (EGFR receptor). It has been found that both mAb-GlcNAc and mAb-GlcNAc(F) were suitable substrates for both EndoSd-D232M and EndoSz-D234M. The ADCC assay of related products, OBI-888-G2S2 and Herceptin-G2S2, showed that the remodeled homogeneous antibody, mAb-G2S2, has an increased relative activity ranging from 3 to 26 folds.



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## GLYCOSYNTHASE VARIANTS FOR GLYCOPROTEIN ENGINEERING AND METHODS OF USE

### CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Patent Application No. 62/690,669, filed on June 27, 2018, the disclosure of all of which are incorporated by reference herein in their entirety.

### FIELD

[0002] The present disclosure relates to glycosynthase enzymes for homogeneous antibody engineering/remodeling. EndoSd-D232 and EndoSz-D234 mutants contain the glycan conjugation enzymatic activity at the conserved N297 glycosylation site of Fc region. The present disclosure demonstrated that glycosynthase activities of EndoSd and EndoSz mutants can be applied on various mAbs targeting different receptors, including OBI-888 (Globo H ganglioside), Herceptin (Her 2 receptor), Perjeta (Her 2 receptor), Erbitux (EGFR receptor), Rituxan (CD20 receptor), OBI-898 (SSEA4 ganglioside), Vectibix (EGFR receptor), Humira (TNF $\alpha$  inactivating), Keytruda (PD-1) and Bavencio (PD-L1). Both mAb-GlcNAc and mAb-GlcNAc(F) were suitable substrates for both EndoSd-D232M and EndoSz-D234M. The ADCC assay of related products showed that the remodeled homogeneous antibody (mAb-G2S2) increased relative activity by several folds.

### BACKGROUND

[0003] Numerous surface carbohydrates are expressed in malignant tumor cells. For example, the carbohydrate antigen Globo H (Fuc $\alpha$ 1 $\rightarrow$ 2 Gal $\beta$ 1 $\rightarrow$ 3 GalNAc $\beta$ 1 $\rightarrow$ 3 Gal $\alpha$ 1 $\rightarrow$ 4 Gal $\beta$ 1 $\rightarrow$ 4 Glc) was first isolated as a ceramide-linked Glycolipid and identified in 1984 from breast cancer MCF-7 cells. (Bremer E G, *et al.* (1984) J Biol Chem 259:14773-14777). Previous studies have also shown that Globo H and stage-specific embryonic antigen 3 (Gal $\beta$ 1 $\rightarrow$ 3GalNAc $\beta$ 1 $\rightarrow$ 3Gal $\alpha$ 1 $\rightarrow$ 4Gal $\beta$ 1 $\rightarrow$ 4Glc $\beta$ 1) (SSEA-3, also called Gb5) were observed on breast cancer cells and breast cancer stem cells (WW Chang *et al.* (2008) Proc Natl Acad Sci USA, 105(33): 11667-11672). In addition, SSEA-4 (stage-specific embryonic antigen-4) (Neu5Ac $\alpha$ 2 $\rightarrow$ 3Gal $\beta$ 1 $\rightarrow$ 3GalNAc $\beta$ 1 $\rightarrow$ 3Gal $\alpha$ 1 $\rightarrow$ 4Gal $\beta$ 1 $\rightarrow$ 4Glc $\beta$ 1) has been commonly used as a cell surface marker for pluripotent human embryonic stem cells and has been used to isolate mesenchymal stem cells and enrich neural progenitor cells (Kannagi R *et al.* (1983) EMBO J, 2:2355-2361). These findings support that Globo series antigens (Globo

H, SSEA-3 and SSEA-4) can be unique targets for cancer therapies and may be used to direct therapeutic agents to targeting cancer cells effectively.

**[0004]** Program death 1 (PD-1) is an inhibitory receptor expressed on T cells, B cells, or monocytes (Ishida *et al.* (1992) *EMBO J.* 11: 3887-2895; Agata *et al.* (1996) *Int. Immunol.* 8: 765-772). PD-L1 and PD-L2 are ligands for PD-1 which have been identified to downregulate T cell activation and cytokine secretion upon binding to PD-1 (Freeman *et al.* (2000) *J Exp Med* 192:1027-34; Latchman *et al.* (2001) *Nat Immunol* 2:261-8). Engagement of PD-1 with PD-L1 or PD-L2 leads to down-regulation of immune responses. Hence, blocking of the PD-1/PD-L1 pathway has been proposed to attenuate central and peripheral immune responses against cancer. Targeting PD-1 and PD-L1 pathway have shown the clinical efficacy in more than 15 cancer types including melanoma, non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC), bladder carcinoma and Hodgkin's lymphoma (Sharma *et al.* (2015) *Science* 348(6230):56-61). However, many patients still fail to respond; in some cases, patients showed initial responses but acquire resistance over time. Therefore, there is an urgent need to identify mechanisms of resistance for combination therapy.

**[0005]** Therapeutic monoclonal antibodies (mAbs) have been developed for the treatment of many diseases, such as cancer, autoimmune, and infectious (Adams, G. P., and Weiner, L. M. (2005) *Nat. Biotechnol.* 23: 1147-1157; Aggarwal, S. R. (2012) *Nat. Biotechnol.* 30: 1191-1197; Aggarwal, S. R. (2014) *Nat. Biotechnol.* 32:323-330). For cancer therapy, several commercial mAbs have been found on the market. Those mAbs recognize particular biomarkers on tumor cell surface and enhance cell apoptosis by different mechanisms, such as turn on antibody-dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) or blocking signal pathway. Her 2 receptor is the most famous biomarker overexpressed on breast cancer, which enable Roche Company to develop two related mAbs, Herceptin (trastuzumab) and Perjeta (pertuzumab). EGFR receptor is also a well-known target for mAb development. For example, Vectibix (panitumumab) and Erbitux (cetuximab) have been developed by Amgen and Merck Company, respectively, targeting metastatic colorectal cancer therapy. In addition, Rituxan (rituximab, Roche) and Arzerra (ofatumumab, GSK) designed to recognize CD20 receptor are commonly used to treat non-Hodgkin's B-cell lymphomas and chronic lymphocytic leukemia cancer. Most recently, OBI Pharma. Inc. is developing OBI-888 and OBI-898 antibodies base on the ganglioside biomarkers Globo H and SSEA-4, which are found in breast, lung, ovary, stomach and small-cell lung (Hakomori, S. I. (2008) *Biochim. Biophys. Acta.* 1780: 325-346; Hakomori, S. and Zhang, Y. (1997) *Chem. Biol.* 4:97-104; Zhang, S. *et al.* (1997) *Int. J. Cancer* 73: 42-49; Zhang, S. *et al.* (1997) *Int. J.*

Cancer 73: 50–56.) but not detectable in normal cell. Erbitux, Rituxan, Arzerra, OBI-888 and OBI-898 kill cancer cells through cytotoxicity in terms of ADCC and CDC. Moreover, several mAbs have been developed to block the function of protein-protein interaction. For example, Humira (adalimumab, AbbVie) blocks the TNF- $\alpha$  receptor mediate signal pathway for autoimmune disease, rheumatoid arthritis; Keytruda (pembrolizumab, Merck) blocks PD-1 receptor to destroy the protective mechanism of cancer cells that treats metastatic melanoma. Compare with small molecular drugs, mAbs are more specific to the target cells and have relatively smaller side-effect to the patients. These two important features have become powerful tools against various diseases.

**[0006]** Monoclonal antibodies (mAbs) have molecular weight ~150 kDa composed of two heavy chains (~50 kDa) and two light chains (~25 kDa), which form three domains separated by flexible hinge region. Two Fab domains contain variable complementarity-determining region (CDR) for identifying antigen. One Fc domain is a constant region with N-glycan for mediation of ADCC and CDC cytotoxicity (Jefferis, R. (2009) *Nat. Rev. Drug Discov.* 8:226–234). The amino acid N297 at the Fc domain is a conserved N-glycosylation site which connects with heterogeneous glycan types, such as biantennary (M3, G0F, G1F, G2F, G0, G1 and G2 complex type) and triantennary (high-mannose and hybrid types), while expressed in various cell systems. The X-ray structure analysis results indicated that the core fucose of Fc glycan obstructed the particular carbohydrate-carbohydrate interactions between Fc and Fc $\gamma$ RIIIa and, as a result, decreased the binding constant for approximately hundred folds (Ferrara, C. *et al.* (2011) *Proc. Natl. Acad. Sci. USA* 108: 12669–12674) and, as a result, reduced cell killing efficiency. The most common cell system used in the industry is CHO cell. CHO cell generally produce the mAbs that contain glycan compositions predominately in the form of G0F, G1F and G2F. These glycan forms limit the activity of mAbs due to the reduced ADCC binding efficiency caused by fucose. Although modified CHO cell systems have been available by FUT8 ( $\alpha$ -1,6-fucosyltransferase 8) gene knock-out (Yamane-Ohnuki, N. and Satoh, M. (2009) *MAbs*, 1: 230–236; Yamane-Ohnuki, N. *et al.* (2004) *Biotechnol. Bioeng.* 87: 614–622) or up-regulation of bisecting GlcNAc (*N*-acetylglucosamine) transferase GnT-III (Umana, P. *et al.* (1999) *Nat. Biotechnol.* 17: 176–180) to reduce the glycan complicity of mAbs, to find a better and more general method to obtain the desired *N*-glycan on mAbs is still in great interests. In addition, it has also reported that removing the Fc glycan will result in losing the ADCC activity (Kurogochi, M. *et al.* (2015) *PLoS One* 10: e0132848). According to the above observations, mAbs cytotoxicity can be effectively controlled by the N-glycan type attached on the Fc region.

**[0007]** Enzymatic modification of Fc region is a solution to establish homogeneous mAbs. Few years ago Lai-Xi Wang and coworkers have tried the chemoenzymatic remodeling by removing the glycan mixture and conjugating homogeneous glycans (Huang, W. *et al.* (2012) *J. Am. Chem. Soc.* 134: 12308–12318). Several endo- $\beta$ -*N*-acetylglucosaminidases (ENGases) have been reported to remove glycan mixture on mAbs. For instance, EndoD (Tai, T. *et al.* (1975) *J. Biol. Chem.* 250: 8569–8575), EndoH (Tarentino, A. L. *et al.* (1974) *J. Biol. Chem.* 249: 818–824), EndoLL (Kuroguchi, M. *et al.* (2015) *PLoS One* 10: e0132848) and EndoM (Kadowaki, S. *et al.* (1990) *Agric. Biol. Chem.* 54: 97–106) are able to hydrolyze the glycan with high mannose or terminal mannose types. EndoS (Collin M and Olsén A. (2001) *EMBO J.* 20: 3046–3055) and EndoSd (Shadnezhad, A. *et al.* (2016) *Future Microbiol* 11: 721–736.) have the ability to hydrolyze non-fucosylated and fucosylated *N*-glycans on the Fc domain, but not high-mannose types. So far, none of signal endoglycosidase could completely hydrolyze all glycan types on mAbs. The EndoS crystal structure was recently solved, which revealed five functional domains (Trastoy, B. *et al.* (2014) *Proc. Natl. Acad. Sci. USA* 111: 6714–6719), in which the endoglycosidase domain is highly conserved with rigid  $\beta$ -barrel structure that suitable for site mutation studies. On the other hand, glycosynthases for antibody Fc were also reported. EndoD-N322Q (Fan, S. Q. *et al.* (2012) *J. Biol. Chem.* 287: 11272–11281) and EndoM-N175Q (Umekawa, M., Li, C. *et al.* (2010) *J. Biol. Chem.* 285: 511–521) only transferred short chain complex-type *N*-glycan to Fc. Endo-F3-D165Q (Giddens, J. P. *et al.* (2016) *J. Biol. Chem.* 291: 9356–9370) only transferred glycan to the fucosylated Fc domain. EndoS-D233Q (Huang, W. *et al.* (2012) *J. Am. Chem. Soc.* 134: 12308–12318) enables conjugation of various bi-antennary complex type, whereas EndoS2-D184M (Li., T., Tong, *et al.* (2016) *J. Biol. Chem.* 291: 16508–16518) has wild substrates including complex, high-mannose and hybrid types.

### SUMMARY OF INVENTION

**[0008]** The present disclosure relates to selected variants of endoglycosidase from *Streptococcus* that possess/display improved enzymatic activities for the synthesis of glycoproteins and/or glycopeptides comprising broad range of well-defined *N*-glycans of high mannose, hybrid and complex types. In particular, one or more embodiments of present invention also relate to use of these enzyme variants for efficient glycan remodeling of therapeutic antibodies to form homogenous glycan compositions at the Fc-domain for improved of their effector functions.

[0009] In the present disclosure, two glycosynthase enzyme variants are provided, EndoSd-D232M and EndoSz-D234M, which have the glycosynthase activity that enable the production of homogeneous mAbs remodeling. Besides the previously reported EndoSd from *Streptococcus dysgalactiae subsp. Dysgalactiae* (NCBI GenBank accession No.: ANI26082.1), we have identified and isolated novel enzymes by protein BLAST database on the EndoS and EndoSd sequences. We selected EndoSz from *Streptococcus equi subsp. Zooepidemicus Sz105* (NCBI GenBank accession No.: KIS14581.1) as another candidate despite it is a putative protein. We generated EndoSd and EndoSz mutants according to multiple sequence alignment. The results showed that both mutated enzymes have unexpectedly improved/enhanced glycosynthase activity to conjugate bi-antennary complex-type glycan to mAbs. The mAbs suitable for conjugation are obtained/derived from a wide-range targets of various biomarkers or different IgG types. We demonstrated that the conjugation results of OBI-888, Herceptin, Perjeta, Erbitux, Rituxan, OBI-898, Vectibix, Humira, Keytruda and Bavencio were satisfactory. We also demonstrated the enzyme efficiency in the conjugation reaction and compared the ADCC activities between heterogeneous and homogeneous mAbs in the related cell systems.

[0010] SEQ ID NO 1:

Name: EndoSd-D232M amino acid sequence

Organism: *Streptococcus dysgalactiae subsp. Dysgalactiae*

MGTILGTHHDSLISVKAEEKITQVSQTSTSIDDLHYLSENSKKEFKEELSKEKVPEKVK  
 EILSKAQQANKQAQELAEMKVPDKIPMKPLNGPLYGGYFRTWHDKTSDPLEKDKV  
 NSMGELPKVDLAFVFHDWTKDYSLFWKELATKHVPKLNKQGTRVIRTIPWRFLAG  
 GDNSGIAEDASKYPNTPEGNKALAKAIVDEYVYKYNLDGLDVMIEHDSIPKVN  
 GEASDENLKR SIDVFEEIGKLIGPKGADKSRLFIMDSTYMADKNPLIERGAPYIDLLLVQV  
 YGSQGEQGEFQNDTKSVTKTPEERWQGYSKYIRPEQYMIGFSFYEEKAGSGNLWYD  
 INARKDEDTANGINDDITGTRAERYARWQPKTGGVKGGIFS YAIDRDGVAHQPKQIA  
 EKDKQSVKNNRPLISEITDNIFHSNYSVSKTLKTVMLKDKAYDLIDEKDFPKALREA  
 VMAQVGRKGDLEFRNGTLRLDNPAIQSLEGLNKFKKLAQLDLIGLSRIIKLDQSVLP  
 ANMKPGKDPLETVLETYKKNNGKEEPAIPVSLTVSGLTGLKELDLSGFDRETLA  
 GID AATLTSLEKVDISDNKLDLAPKTENRQIFDVMLSTVNNNAGISEQSIKFDNQPAGN  
 YPQTYGATNLQLPVRQEKIDLQHQLLFGTITNQGTLINSEADYKTYRNQKIAGRNFV  
 DPDYPPYNNFKVSHDNYTVKVTDSLGTITTDKMLATDKEETYKVDFFSPTDKTKAVH  
 TAKVIVGDEKTMVMVLAEGATVIKSENDENAQKVFNGIMEYNPLSFNNKSSIFEIKD  
 PSLAKYWRLFNDSSKDKKDYIKEAKLEVFTGQLNAEADVKTILEKPDNWWTVSTYS  
 GEEKVFSHSLDNISAKYWRVTVDNKKDQYGYVSLPELQILGYPLPNADTIMKT VTV  
 AKELSQQKDKFPQQLDESTAKEAVVEASLNSKLFDTGVINTNVEALKNVVDECLA  
 YEKNKETAFKATEDYRAAVNGVKAESVTVEEMAQLKDLIGKAAHLNSKIDAKLAD  
 REYDKDLLGLIGELTNITRTVKS FVKHHHHHHH

[0011] SEQ ID NO 2:

Name: EndoSz-D234M amino acid sequence

Organism: *Streptococcus equi subsp. Zooepidemicus Sz105*

MVAILAAQHDSLIRVKAEDKLVQTSPSVSAIDALHYLSENSKKEFKEELSKVEKAQP
EKLKEIVSKAQQADKQAKTLAEMKVPEKIPMKPLKGPLYGGYFRTWHDKTSDDPAEK
DKVNSMGELPKEVDLAFVFDHWTKDYSFLWQELATKHVPTLNKQGTRVIRTIPWRF
LAGGDHSGIAEDAQKYPNTPEGNKALAKAIVDEYVYKYNLDGLDVMIERDSIPKVN
KEESKEGIERSIQVFEEIGKLIGPKGADKSRLFIMDSTYMADKNPLIERGAPYIDLLLV
QVYGTQGEKGGFDNANHKAVDTMEERWESYSKYRPEQYMVGFsfyEEKANSGLN
WYDVNVEDDTNPNIGSEIKGTRAERYAKWQPKTGGVKGIFSYGIDRDGVAHPKKN
GPKTPDLDKIVKSDYKVSALKKVMENDKSYELIDQKDFPDKALREAVIAQVGSRR
GNLERFNGTLRLDNPDIKSLEGLNKLKLALELIGLSQITKLDSSVLPENIKPTKDTL
VSVLETYKNDDRKEEAKAIPQVALTISGLTGLKELNLAGFDRDSLALGIDAASLTSLEK
VDLSSNKLDLAAGTENRQILDTMLATVTKHGGVSEKTFVFDHQKPTGLYPDTYGTK
SLQLPVANDTIDLQAKLLFGTVTNQGTLINSEADYKAYQEQEIAGHRFVDSYDYKA
FAVTYKDYKIKVTDSTLGVTDHKDLSTKEETYKVEFFSPINSTKPVHEAKIVVGEER
TMMVNLAEGATIIGGDADPTNAKKVFDGLLNNDTTTLSTSNKASIIFELKEPGLVKH
WRRFNDSKISKADYIKEAKLEAFVGHLEDSSKVKDSLEKSTEWVTVSDYSGEAQEFS
QPLNNGAKYWRITDNKKSQYGYVSLPELQIIGHRLPEAATVMTTMAAAEELSQQK
DKFSQEQLELEVKVAALKAALDNKMFNADTINASFADVKAYIDKLLADAAGKKT
LGKATKEAQPVAATDAKEKAESENPKADHHHHHH

[0012] SEQ ID NO 3:

Name: EndoSd-D232M nucleic acid sequence

Organism: *Streptococcus dysgalactiae subsp. Dysgalactiae*

ATGGGCACCATCCTGGGTACCCACCACGACAGCCTGATCAGCGTGAAGGCGGAG
GAAAAAATTACCCAAGTTAGCCAAACCAGCACCAGCATTGACGATCTGCACTAC
CTGAGCGAAAACAGCAAGAAAGAGTTCAAAGAGGAGCTGAGCAAGGAGAAAGT
GCCGAAAAGGTTAAAGAGATCCTGAGCAAAGCGCAGCAAGCGAACAAGCAGG
CGCAAGAGCTGGCGGAAATGAAGGTGCCGACAAAATTCCGATGAAGCCGCTGA
ACGGTCCGCTGTATGGTGGCTACTTTCGTACCTGGCACGACAAAACCAGCGATCC
GCTGGAAAAGGACAAAGTTAACAGCATGGGCGAACTGCCGAAAGAGGTGGATC
TGGCGTTCGTTTTTACGACTGGACCAAAGATTATAGCCTGTTCTGGAAAGAGCT
GGCGACCAAGCACGTGCCGAAGCTGAACAAAACAGGGTACCCGTGTTATCCGTAC
CATTCCGTGGCGTTTTCTGGCGGGTGGCGACAACAGCGGTATTGCGGAAGATGC
GAGCAAGTACCCGAACACCCCGGAGGGTAACAAAAGCGCTGGCGAAGGCGATTGT
GGACGAATACGTTTATAAATAACAACCTGGACGGTCTGGATGTGATGATCGAGCA
CGATAGCATTCCGAAAGTTAACGGCGAAGCGAGCGACGAGAACCTGAAGCGTAG
CATCGATGTGTTTCGAGGAAATCGGTAAACTGATTGGTCCGAAAGGCGCGGACAA
GAGCCGTCTGTTTATTATGGACAGCACCTATATGGCGGATAAGAACCCGCTGATC
GAACGTGGCGCGCCGTATATTGACCTGCTGCTGGTGCAGGTTTACGGTAGCCAGG
GCGAGCAGGGTGAATTCCAAAACGATACCAAAGCGTTACCAAGACCCCGGAGG
AACGTTGGCAGGGCTATAGCAAATACATCCGTCCGGAGCAATATATGATTGGTTT
CAGCTTTTACGAGGAAAAGGCGGGTAGCGGCAACCTGTGGTACGACATCAACGC
GCGTAAAGACGAAGATAACCGCGAACGGCATCAACGACGATATTACCGGTACCCG

TGCGGAGCGTTATGCGCGTTGGCAGCCGAAAACCGGTGGCGTGAAGGGTGGCAT  
 CTTTAGCTACGCGATTGACCGTGATGGTGTTCGCGACCAGCCGAAGCAAATCGCG  
 GAAAAGGACAAACAAAGCGTGAAAAACAACCGTCCGCTGATCAGCGAGATTAC  
 CGATAACATTTTCCACAGCAACTATAGCGTGAGCAAGACCCTGAAAACCGTTAT  
 GCTGAAGGACAAAGCGTACGACCTGATCGATGAAAAAGACTTTCCGGATAAAGC  
 GCTGCGTGAGGCCGGTGTATGGCGCAGGTTGGCACCCGTAAGGGTGACCTGGAACG  
 TTTCAACGGCACCCCTGCGTCTGGATAACCCGGCGATCCAGAGCCTGGAGGGTCTG  
 AACAAAGTTTAAGAACTGGCGCAACTGGACCTGATTGGCCTGAGCCGTATCATT  
 AAAGTGGATCAAAGCGTGCTGCCGGCGAACATGAAGCCGGGTAAAGACCCGCTG  
 GAAACCGTTCTGGAGACCTACAAGAAAAACGGCAAAGAGGAGCCGGCGATCATT  
 CCGCCGGTTAGCCTGACCGTTAGCGGTCTGACCGGTCTGAAAGAAGTGGACCTG  
 AGCGGCTTCGATCGTGAGACCCTGGCGGGTATCGATGCGGCGACCCTGACCAGC  
 CTGGAAAAGGTGGACATTAGCGATAACAAACTGGACCTGGCGCCGAAGACCGAG  
 AACCGTCAGATCTTCGATGTGATGCTGAGCACCGTTAACAACAACGCGGGTATC  
 AGCGAGCAGAGCATTAAATTTGACAACCAAAAAGCCGGCGGGCAACTATCCGCAA  
 ACCTACGGTGCACCAACCTGCAGCTGCCGGTTCGTCAAGAAAAAATCGACCTG  
 CAGCACCAACTGCTGTTCCGGCACCATCACCAACCAGGGTACCCTGATTAACAGC  
 GAGGCGGATTATAAAAACCTACCGTAACCAAAAAGATTGCGGGTCTGTAACCTCGTG  
 GACCCGGATTATCCGTACAACAACCTTAAAGTTAGCCACGACAACCTATACCGTGA  
 AGGTTACCGATAGCACCCCTGGGCACCACCACCGACAAAATGCTGGCGACCGATA  
 AAGAGGAAACCTACAAGGTGGACTTCTTTAGCCCGACCGATAAGACCAAAGCGG  
 TTCACACCGCGAAAGTGATCGTTGGCGACGAAAAGACCATGATGGTGAACCTGG  
 CGGAGGGTGCACCGTTATCAAAGCGGAGAACGATGAAAACGCGCAGAAGGTTT  
 TCAACGGTATTATGGAATATAACCCGCTGAGCTTCAACAACAAGAGCAGCATCA  
 TTTTGTAGATCAAAGACCCGAGCCTGGCGAAGTATTGGCGTCTGTTCAACGATAG  
 CAGCAAAGACAAGAAAGATTACATCAAGGAAGCGAAACTGGAAGTGTTTACCG  
 GTCAGCTGAACGCGGAAGCGGACGTTAAAACCATCTGGAGAAGCCGGATAACT  
 GGGTGACCGTTAGCACCTATAGCGGCGAGGAAAAGGTGTTTAGCCACAGCCTGG  
 ACAACATCAGCGCGAAATACTGGCGTGTGACCGTTGACAACAAGAAAGATCAGT  
 ATGGCTACGTTAGCCTGCCGGAGCTGCAAATCCTGGGTTACCCGCTGCCGAACGC  
 GGATAACATTATGAAAACCGTGACCGTTGCGAAGGAACTGAGCCAGCAAAGGA  
 CAAATTCGCGCAGCAACTGCTGGATGAGAGCACCGCGAAGGAAGCGGTGGTTGA  
 GCGGAGCCTGAACAGCAAACCTGTTTGACACCGGTGTGATCAACACCAACGTTGA  
 AGCGCTGAAGAACGTGGTTGATGAGTGCCTGGCGTATGAAAAGAACAAGAGAC  
 CGCGTTCAAGGCGACCGAAGACTACCGTGCGGCGGTGAACGGTGTAAAGCGGA  
 GAGCGTGACCGTTGAGGAAATGGCGCAGCTGAAAGATCTGATCGGCAAGGCGGC  
 GCACCTGAACAGCAAATGACGCGAAGCTGGCGGATCGTGAATACGACAAAGA  
 TCTGCTGGGCCTGATCGGCGAGCTGACCAACATTACCCGTACCGTGAAGAAGCTTT  
 GTTAAGTGA

[0013] SEQ ID NO 4:

Name: EndoSz-D234M nucleic acid sequence

Organism: *Streptococcus equi subsp. Zooepidemicus Sz105*

ATGGTTGCGATCCTGGCGGGCGCAACACGATAGCCTGATTCGTGTGAAGGCGGAG  
 GACAAACTGGTGCAGACCAGCCCGAGCGTTAGCGCGATTGATGCGCTGCACTAC  
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AATGTTCAACGCGGACACCATCAACGCGAGCTTTGCGGACGTTAAAGCGTACAT  
TGATAAGCTGCTGGCGGACGCGGCGGGTAAGAAAACCCCTGGGCAAAGCGACCA

AAGAGGCGCAGCCGGTGGCGACCGATGCGAAGGAAAAAGCGGAGAGCGAAAAC  
CCGAAGGCGGACTAA

[0014] In one aspect, the present invention provides the glycosynthase enzymes variants, wherein the variants have at least about 80%, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% sequence and/or structural homology thereto and exhibit improved transglycosylation activity on both fucosylated and non-fucosylated GlcNAc acceptors against broad range of N-glycans of high mannose, hybrid and complex types, wherein the said variants enable efficient transfer of an activated oligosaccharide donors on fucosylated and non-fucosylated GlcNAc acceptors to form new homogenous glycoform of glycopeptide or glycoprotein or therapeutic antibodies. The EndoSz and EndoSd mutants are listed in the following table:

| EndoSz mutant | EndoSd mutant |
|---------------|---------------|
| D234E         | D232E         |
| D234R         | D232R         |
| D234H         | D232H         |
| D234M         | D232M         |
| D234V         | D232V         |
| D234L         | D232L         |
| D234F         | D232F         |
| D234S         | D232S         |
| D234T         | D232T         |
| D234Q         | D232Q         |
| T183Q         | T181Q         |
| D232Q         | D230Q         |
| D280Q         | D278Q         |
| S281Q         | S279Q         |
| T282Q         | T280Q         |

[0015] In one aspect, the disclosure provides vectors and cells lines suitable for expressing the peptides of SEQ IDs No 1 or No. 2. In one embodiment, the vector or cell line comprises the nucleotide of SEQ ID No 3 or No. 4.

[0016] In one aspect, that the sequence and/or structural homologs can include or exclude natural sequences.

[0017] In certain embodiment, the antibody is OBI-888 (Anti-Globo H monoclonal antibody). Exemplary OBI-888 is as described in PCT patent publications (WO2015157629A2 and WO2017062792A1), patent applications, the contents of which are incorporated by reference in its entirety.

[0018] In certain embodiment, the antibody is OBI-898 (Anti-SSEA4 monoclonal antibody). Exemplary OBI-898 is as described in PCT patent publication (WO2017172990A1), patent applications, the contents of which are incorporated by reference in its entirety.

[0019] In another aspect, a method for preparing an engineered glycoprotein using the glycosynthase variants is provided.

[0020] In another aspect, a population of homogeneous antibody prepared by using the glycosynthase variants is provided.

### BRIEF DESCRIPTION OF THE DRAWINGS

[0021] **Figure 1.** Summarizes aspects of the overall process of homogeneous platform. (A) The cell expressed mAbs were heterogeneous with glycan mixtures which were removed by wild type EndoSz and  $\alpha$ -fucosidase to generate mAb-GlcNAc. Then the EndoSd-D232M and EndoSz-D234M were used to conjugate glycan-oxazoline and produce homogeneous mAbs. (B) In the glycan cleavage step, only EndoSz enzyme is used to generate mAbs-GlcNAc-F and the product will be homogeneous-mAb-glycan-F after conjugation. (C) The exemplary picture of biantennary glycans.

[0022] **Figure 2.** LC/MS/MS results of Herceptin-GlcNAc glycopeptide. The Herceptin wild type was mixed with EndoSz wild type and  $\alpha$ -fucosidase to remove the glycans on the Fc region. The result showed all of the glycans were removed and generated >99% Herceptin-GlcNAc.

[0023] **Figure 3.** The exemplary detection methods for homogeneous platform (demonstrated by EndoSz-D234M). (A) The HPLC analysis method. Original Herceptin (green) had retention time 12.5 minute, and Herceptin-GlcNAc (magenta) shifted to 11.4 minute. In the transglycosylation of NSCT-oxa, the Herceptin-G2S2 (blue) could be clearly distinguished by 1N-G2S2 (hemi-glycosylated) and 2N-G2S2 (fully glycosylated) with retention time 12.6 and 13.9 minute, respectively. In the transglycosylation process, the peaks sequentially shifted from Herceptin-GlcNAc to Herceptin-1N-G2S2 and Herceptin-2N-G2S2,

which allowed us to monitor the process. (B) The SDS-PAGE result for comparison. Hemi-glycosylated and fully glycosylated Herceptin-G2S2 could not be easily identified.

**[0024]** **Figure 4.** The multiple sequence alignment of exemplary EndoS2, EndoS, EndoSz and EndoSd. The blue triangle indicated the selected sites for site-direct mutagenesis study in EndoSz and EndoSd enzymes.

**[0025]** **Figure 5.** The time dependent transglycosylation results (A) The transglycosylation of NSCT-oxa and Herceptin-GlcNAc with molar ratio 20:1 by EndoSz-D234M. (B) EndoSd-D232M used higher molar ratio 150:1 (NSCT-oxa : Herceptin-GlcNAc). The reaction was started with 100% Herceptin-GlcNAc. According to the efficiency, the percentage of Herceptin-1N-G2S2 and Herceptin-2N-G2S2 were formed. Transglycosylation efficiency of >90% Herceptin-2N-G2S2 could be reached in both enzymes.

**[0026]** **Figure 6.** The relative transglycosylation activities in different exemplary mutants. (A) EndoSz (B) EndoSd.

**[0027]** **Figure 7.** Demonstration of efficacy: The ADCC assay results. (A) The ADCC result of Herceptin and Herceptin-G2S2. The data showed Herceptin had higher ADCC activity. The EC<sub>50</sub> of Herceptin and Herceptin-G2S2 was 15.29 (µg/mL) and 5.10 (µg/mL), respectively. (B) The summary of the transglycosylation efficiency and ADCC in various mAbs.

## Abbreviations

**[0028]** ACN: acetonitrile; ADCC: antibody-dependent cellular cytotoxicity; CDC: complement dependent cytotoxicity; CDR: complementarity-determining region; FA: Formic acid; FUT8:  $\alpha$ -1,6-fucosyltransferase 8; GlcNAc: N-acetylglycosamine; HFIP: 1,1,1,3,3,3-Hexafluoro-2-propanol; IPTG: isopropyl- $\beta$ -D-thiogalactopyranoside; TFA: trifluoroacetic acid.

## DETAILED DESCRIPTION OF THE INVENTION

**[0029]** N-glycosylation is one of the most complex post-translational modifications that often result in a remarkable heterogeneity of glycan structures including high mannose, hybrid and complex types, depending on the recombinant expression system. Commercially available therapeutic antibodies typically exist as mixtures of glycoforms that are not optimal for their respective therapeutic activities. Recently, glycoengineering has gathered attention to control Fc glycosylation for improving efficacy.

**[0030]** A typical IgG consists of two antigen-binding fragments (Fabs), which are connected via a flexible region to a constant region (Fc). The Fab domains are responsible for

antigen recognition while the N-glycan at Asn297 of Fc domain interact with respective Fc receptors (such as FcγRIIIa and FcγRIIb) on effector cells and C1q component of the complements that activate the effector functions, including antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Almost all therapeutic antibodies are N-glycosylated on each of the homodimeric Fc domains at the conserved asparagine residue (N297). These N-linked glycans result in more than 30 different glycoforms, and are typical biantennary complex type with considerable structural heterogeneity, in which the core heptasaccharide can be differentially decorated with core fucose (Fuc), bisecting N-acetylglucosamine (GlcNAc), terminal galactose (Gal), and terminal sialic acid (Sia). The composition of N-glycans could influence the Fc domain conformation, therefore, modulating the antibody's stability, pharmacokinetic profile, immunogenicity, effector functions, antibody-mediated inflammation, and complement activation. For example, the absence of the core fucose, as well as the attachment of a bisecting GlcNAc moiety, dramatically enhances the affinity of antibody for the FcγRIIIa receptor (FcγRIIIa) on effector cells, resulting in more effective elimination of the target. In addition, the terminal α-2,6-sialylated glycan, which is a minor component of antibodies and the intravenous immunoglobulin (IVIG), is an optimized structure that enhances the anti-inflammatory properties.

**[0031]** Endoglycosidases, are a family of at least 18 glycoside hydrolase (GH) from the *Streptococcus pyogenes* and have recently become the point of attention for glycoengineering of therapeutic antibodies. These enzymes can catalyze the hydrolysis of the β-1, 4 linkage between the two N-acetylglucosamines (GlcNAcs) in the core of the N-linked glycan of human IgG. Additionally, the enzymes remove complex type glycans at IgG Fc domain.

**[0032]** Embodiments of the present disclosure relate to selected variants of glycosynthase that show remarkable transglycosylation activities to transfer a broad range of N-glycans of high mannose, hybrid or complex types, from activated oligosaccharide oxazolines to fucosylated or non-fucosylated GlcNAc-peptides, proteins or IgGs with little or negligible product hydrolysis. The novel Glycosynthase enzymes acted with surprising efficiency to provide homogeneously glycosylated glycopeptides, glycoproteins and therapeutic antibodies and Fc fragments thereof, having various defined glycoforms. Still further, embodiments of the present invention may provide glycoengineered antibodies with enhancement of their effector functions, such as FcγIIIa bindings and antibody dependent cell mediated cytotoxicity (ADCC) etc., as well as pharmacological properties. Embodiments of the present invention also allow for rapid investigation of effects of diverse Fc glycosylations of therapeutic antibodies on their effector functions.

**[0033]** In accordance with embodiments of the invention, a novel Glycosynthase enzyme comprises a sequence selected from the sequences of SEQ ID NOs. 1-2. These mutants show unexpectedly improved transglycosylation activities and reduced hydrolyzing activities. Therefore, they can catalyze efficient transfer of activated oligosaccharide donors to core GlcNAc-acceptors, which may be fucosylated or non-fucosylated.

**[0034]** In accordance with certain embodiments, a Glycosynthase enzyme may have a sequence identity of at least about 80% (e.g., 80%, 85%, 90%, 95%, or 98% (or a value ranging between any of the two numbers listed herein) to a sequence in SEQ ID Nos. 1-2 and have the desired transglycosylation activity, or fragment thereof having the transglycosylation activity.

### **Definitions**

**[0035]** It must be noted that as used herein and in the appended claims, the singular forms “a”, “an”, and “the” include plural reference unless the context clearly dictates otherwise.

**[0036]** As used herein, the term “glycan” refers to a polysaccharide, oligosaccharide or monosaccharide. Glycans can be monomers or polymers of sugar residues and can be linear or branched. A glycan may include natural sugar residues (e.g., glucose, N-acetylglucosamine, N-acetyl neuraminic acid, galactose, mannose, fucose, hexose, arabinose, ribose, xylose, etc.) and/or modified sugars (e.g., 2'-fluororibose, 2'-deoxyribose, phosphomannose, 6' sulfo N-acetylglucosamine, etc.).

**[0037]** As used herein, the terms “fucose,” “core fucose,” and “core fucose residue” are used interchangeably and refer to a fucose in  $\alpha$ -1,6-position linked to the N-acetylglucosamine .

**[0038]** As used herein, the terms “N-glycan”, “N-linked glycan”, “N-linked glycosylation”, “Fc glycan” and “Fc glycosylation” are used interchangeably and refer to an N-linked oligosaccharide attached by an N-acetylglucosamine (GlcNAc) linked to the amide nitrogen of an asparagine residue in a Fc-containing polypeptide. The term “Fc-containing polypeptide” refers to a polypeptide, such as an antibody, which comprises an Fc region.

**[0039]** As used herein, the term “glycosylation pattern” and “glycosylation profile” are used interchangeably and refer to the characteristic “fingerprint” of the N-glycan species that have been released from a glycoprotein or antibody, either enzymatically or chemically, and then analyzed for their carbohydrate structure, for example, using LC-HPLC, or MALDI-TOF MS, and the like. See, for example, the review in Current Analytical Chemistry, Vol. 1, No. 1 (2005), pp. 28-57; herein incorporated by reference in its entirety.

**[0040]** As used herein, the term “glycoengineered Fc” when used herein refers to N-glycan on the Fc region has been altered or engineered either enzymatically or chemically. The

term “Fc glycoengineering” as used herein refers to the enzymatic or chemical process used to make the glycoengineered Fc.

**[0041]** The terms “homogeneous”, “uniform”, “uniformly” and “homogeneity” in the context of a glycosylation profile of Fc region are used interchangeably and are intended to mean a single glycosylation pattern represented by one desired N-glycan species, with little or no trace amount of precursor N-glycan, including, for example, less than 95, 96, 97, 98, 99 % starting precursor material.

**[0042]** Table 1. Listed the four classes of amino acids.

| Side Chain  | Amino Acid   |
|-------------|--|
| Basic       | Arginine (R), Lysine (K) or Histidine (H)  |
| Neutral     | Cysteine (C), Tyrosine (Y), Glycine (G), Glutamine (Q), Threonine (T), Asparagine (N) or Serine (S)                    |
| Hydrophobic | Isoleucine (I), Leucine (L), Methionine (M), Tryptophan (W), Proline (P), Valine (V), Phenylalanine (F) or Alanine (A) |
| Acidic      | Aspartic Acid (D) or Glutamic Acid (E)   |

**[0043]** As used herein, the terms “IgG”, “IgG molecule”, “monoclonal antibody”, “immunoglobulin”, and “immunoglobulin molecule” are used interchangeably.

**[0044]** As used herein, the term “Fc receptor” or “FcR” describes a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the Fc $\gamma$ RI, Fc $\gamma$ RII, and Fc $\gamma$ RIII subclasses, including allelic variants and alternatively spliced forms of these receptors. Fc $\gamma$ RII receptors include Fc $\gamma$ RIIA (an “activating receptor”) and Fc $\gamma$ RIIIB (an “inhibiting receptor”), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor Fc $\gamma$ RIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor Fc $\gamma$ RIIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain, (see review M. in Daëron (1997) *Annu. Rev. Immunol.* 15:203-234). FcRs are reviewed in Ravetch and Kinet (1991) *Annu. Rev. Immunol.* 9:457-92; Capel *et al.* (1994) *Immunomethods* 4:25-34; Haas *et al.* (1995) *J. Lab. Clin. Med.* 126:330-41). Other FcRs, including those to be identified in the future, are encompassed by the term “FcR” herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer *et al.* (1976) *J. Immunol.* 117:587 and Kim *et al.* (1994) *J. Immunol.* 24:249).

**[0045]** The term “effector function” as used herein refers to a biochemical event that results from the interaction of an antibody Fc region with an Fc receptor or ligand. Exemplary “effector functions” include C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor; BCR), etc. Such effector functions can be assessed using various assays known in the art.

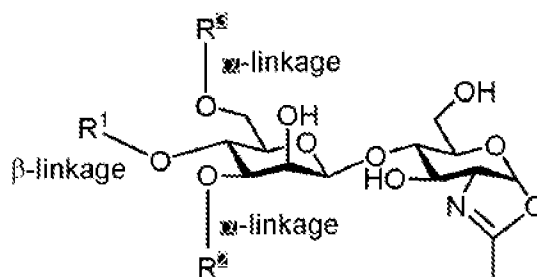
**[0046]** As used herein, the term “Antibody-dependent cell-mediated cytotoxicity” or “ADCC” refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. The antibodies “arm” the cytotoxic cells and are absolutely required for such killing. The primary cells for mediating ADCC, NK cells, express Fc $\gamma$ RIII only, whereas monocytes express Fc $\gamma$ RI, Fc $\gamma$ RII and Fc $\gamma$ RIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in U.S. Pat. No. 5,500,362 or U.S. Pat. No. 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in an animal model such as that disclosed in Clynes *et al.* (1998) PNAS (USA) 95:652-656.

**[0047]** The complex N-linked oligosaccharide on each CH2 domain of IgGs is crucial for the structure of the Fc region and thus the interaction with the Fc receptors (Krapp *et al.* 2003; Woof and Burton 2004). The oligosaccharide chain at IgG-Fc domain contains several N-Acetyl-Glucosamine (GlcNAc) and mannose (Man) residues, and eventually galactose (Gal) and fucose (Fuc) residues as well as sialic acid (Sia or NANA for N-acetylneuraminic acid). A GlcNAc, with or without al-6 Fuc, is attached to the Asn297. A GlcNAc $\beta$ 1-4 is attached to this first GlcNAc. A man $\beta$ 1-4 is then found, to which two Man $\alpha$ 1-6 and Man $\alpha$ 1-3 arms are attached. Both arms contain an additional GlcNAc $\beta$ 1-2 to which a Gal $\beta$ 1-4 can be attached or not. Thus, the carbohydrate chain can contain 0, 1 or 2 galactose residues, defining G0, G1, and G2 glycoforms, respectively. Further variations occur, including the presence of a bisecting GlcNAc $\beta$ 1-4 and the capping of one or both of the terminal galactose residues with a sialic acid or even a Gal $\alpha$ 1-3 residue. The enzymatic cleavage of the Fc-glycan with Endoglycosidases causes the Fc region to deform, and thus, dramatically decrease in IgGs binding to Fc $\gamma$  receptors (Allhorn *et al.* 2008). Despite of their 37% sequence identity, both EndoS and EndoS2 catalyze

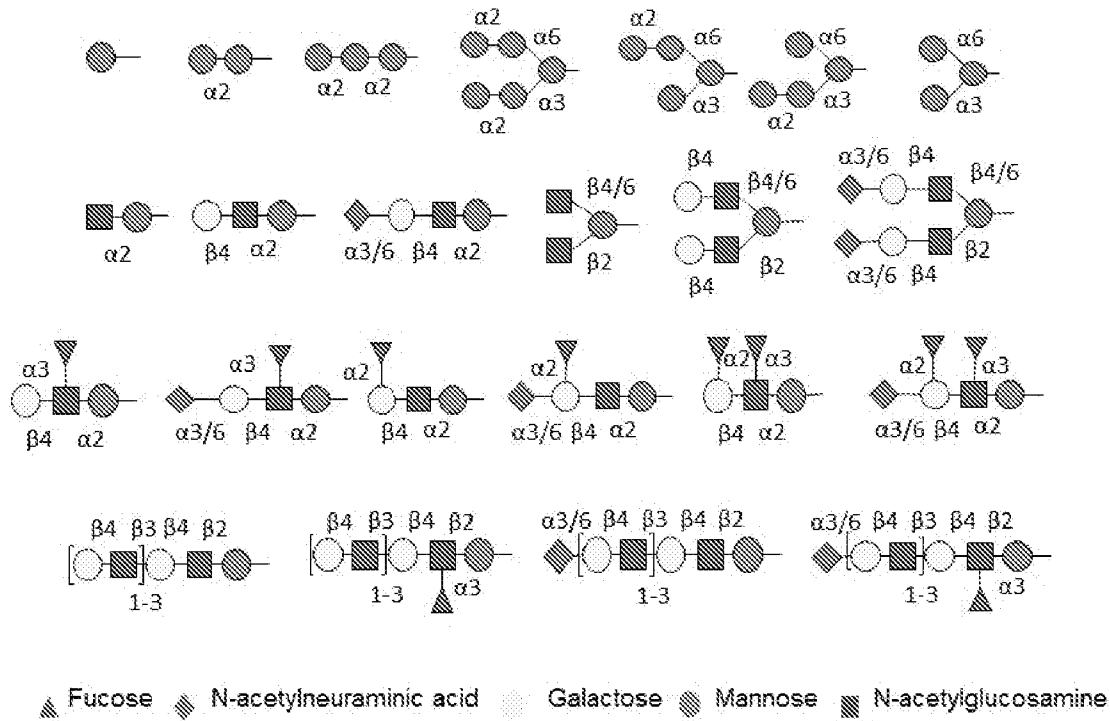
the hydrolysis of the  $\beta$ -1,4 linkage between the two N-acetylglucosamines (GlcNAcs) in the core of the N-linked glycan of human IgG. However, in addition to complex type glycans, EndoS2 hydrolyze hybrid and oligomannose structures to a greater extent compared with EndoS (Sjögren *et al.* 2015).

**[0048]** Since the first antibody therapy was introduced in the 1980s, there are more than 240 therapeutic antibodies in clinical trials and the field is steadily expanding (Chan and Carter 2010). The role of the IgG-Fc glycans on antibody functions has gained a huge attention in the growing field of monoclonal therapeutic antibodies. Therefore, to improve the efficacy of the therapeutic antibodies, the major focus is turning towards the engineering the Fc-glycan that specifically interact with selected Fc $\gamma$  receptors (Sondermann *et al.* 2013; Bournazos *et al.* 2014; Monnet *et al.* 2014; Quast and Lünemann 2014). Some of the important glycan modifications that dramatically affect the effector functions includes, i) the lack of a core fucose residue attached to the reducing end GlcNAc residue leads to increased affinity for Fc $\gamma$  RIIIa and thus increased antibody-dependent cytotoxicity (Iidaet *et al.* 2006); ii) sialic acid rich glycans on IgG that have been claimed to increase the anti-inflammatory response of IgGs through increased interaction with DC-SIGN receptors on dendritic cells and macrophages (Anthony *et al.* 2008; Anthony and Ravetch 2010; Pincetic *et al.* 2014); iii) having bisecting GlcNAc induces a strong ADCC as compared to its parental counterpart. The recent improvements in biotechnology tools to control the Fc-glycosylation states of IgG facilitates development of therapeutic antibodies with predefined glycoforms. Accordingly, the Glycosynthase enzymes of present invention is a great advancement in the field for glyco-engineering of peptides, proteins, and antibodies of interest to attach broad range of N-glycans of high mannose, hybrid and complex types for functional and structural studies.

**[0049]** In one embodiment, the synthetic glycan oxazoline comprising diverse N-glycans of high mannose, hybrid and complex types having the formula:



Wherein, R<sup>1</sup> is -H or N-acetyl glucosamine attached via  $\beta$ -1, 4 linkage and R<sup>2</sup> and R<sup>3</sup> are same or different and are independently selected from the group consisting of:



**[0050]** In another aspect, the present invention provides exemplary Glycosynthase enzymes for transglycosylation at core fucosylated or non-fucosylated GlcNAc-acceptor, wherein the core fucosylated or non-fucosylated GlcNAc-acceptor comprising core fucosylated or non-fucosylated GlcNAc-peptides, proteins and IgG Fc domain or fragment thereof.

**[0051]** In a separate aspect, the present invention provides a remodeling method of core fucosylated or non-fucosylated GlcNAc-peptide, protein, and IgG or IgG-Fc fragment, wherein the method comprising: providing peptide/protein/antibody-GlcNAc acceptor or Fc fragment and reacting with an activated oligosaccharide donors under the catalysis of *Streptococcus dysgalactiae subsp. Dysgalactiae* and *Streptococcus equi subsp. Zooepidemicus Sz105* glycosynthase enzymes, and thereby preparing substantially, essentially, and/or pure glycoforms of pre-existing peptides, proteins and monoclonal antibodies having heterogeneous glycosylation states.

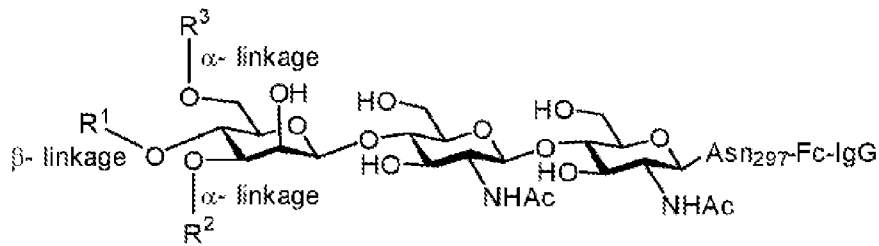
**[0052]** In further aspect, the present invention provides method of using Glycosynthase enzymes for glycan remodeling of therapeutic IgG or Fc fragment thereof, wherein the method comprising:

A. Treating natural or recombinant core fucosylated or non-fucosylated therapeutic IgG or IgG-Fc fragment carrying heterogeneous N-glycans with Endoglycosidase (e.g. wild type EndoS2) together with or without bacterial alpha fucosidases to hydrolyze bond between two

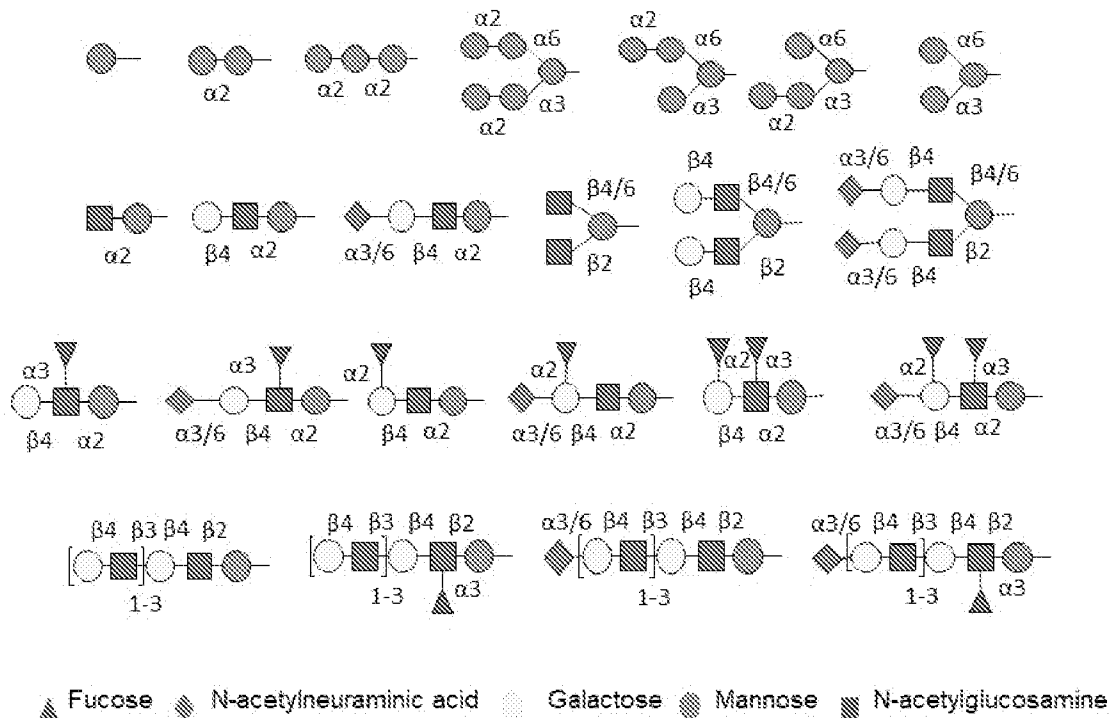
reducing end GlcNAc residues to form core fucosylated or non-fucosylated GlcNAc-IgG acceptor;

B. Transferring the wide range of predefined oligosaccharide building units in the form of activated oligosaccharide donors to core fucosylated or non-fucosylated GlcNAc-IgG to reconstitute natural beta 1, 4 linkage through transglycosylation using *Streptococcus dysgalactiae subsp. Dysgalactiae* and *Streptococcus equi subsp. Zooepidemicus Sz105* Glycosynthase enzymes, thereby attaching the predefined oligosaccharide to remodel core fucosylated or non-fucosylated IgG or Fc fragment thereof.

[0053] In further aspect, the present invention provides a composition of fucosylated or non-fucosylated glyco-engineered antibodies or antigen binding fragments comprising of IgG molecules having the same N-glycan structure at each site of the Fc region, wherein the N-glycan is of high mannose, hybrid, and complex types and is selected from the group consisting of:



Wherein, R<sup>1</sup> is -H or N-acetyl glucosamine attached via β-1, 4 linkage and R<sup>2</sup> and R<sup>3</sup> are same or different and are independently selected from the group consisting of:



[0054] In another aspect, the present invention provides the glycoengineered antibodies with unexpectedly improved effector functions such as bindings to Fc $\gamma$ IIIa, ADCC and regulates immune response, as compared to non-modified antibodies.

[0055] Another aspect of the present disclosure features a pharmaceutical composition comprising a composition of glyco-engineered antibodies described herein and a pharmaceutically acceptable carrier for the treatment of cancer in a patient.

[0056] As used herein, examples of cancers include, but not limited to, cancers associated with and/or expressing Globo series antigens, including, but not limited to, Globo H, SSEA-4, SSEA-3; cancers associated with and/or expressing Her-2; cancers associated with and/or expressing EGFR CD20, TNF- $\alpha$ , PD-1 and PD-L1 receptor.

[0057] The subject to be treated by the methods described herein can be a mammal, more preferably a human. Mammals include, but are not limited to, farm animals, sport animals, pets, primates, horses, dogs, cats, mice and rats. A human subject who needs the treatment may be a human patient having, at risk for, or suspected of having cancer, which include, but not limited to, sarcoma, skin cancer, leukemia, lymphoma, brain cancer, lung cancer, breast cancer, oral cancer, esophagus cancer, stomach cancer, liver cancer, bile duct cancer, pancreas cancer, colon cancer, kidney cancer, cervix cancer, ovary cancer and prostate cancer. A subject having cancer can be identified by routine medical examination. Particularly, the cancer is Globo series antigen expressing cancer.

[0058] The present invention envisioned glycoengineering of antibodies selected from the group consisting of Herceptin (trastuzumab), Perjeta (pertuzumab), Erbitux (cetuximab), Rituxan (rituximab), Vectibix (panitumumab), Humira (adalimumab), Keytruda (pembrolizumab) and Bavencio (avelumab).

### **Pharmaceutical Formulations**

[0059] Therapeutic formulations comprising an antibody of the invention may be prepared for storage by mixing the antibody having the desired degree of purity with one or more optional physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of aqueous solutions, lyophilized or other dried formulations. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, histidine and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride;

hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

**[0060]** The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, including, but not limited to, those with complementary activities that do not adversely affect each other. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

**[0061]** The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacrylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nano-capsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980).

**[0062]** The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

**[0063]** Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the immunoglobulin of the invention, which matrices are in the form of shaped articles, e.g., films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and  $\gamma$  ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated immunoglobulins remain in the body for a long time, they may denature or aggregate as a result

of exposure to moisture at 37 °C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

**[0064]** The amount of antibody in the pre-lyophilized formulation is determined taking into account the desired dose volumes, mode(s) of administration etc. Where the protein of choice is an intact antibody (a full-length antibody), from about 2 mg/mL to about 50 mg/mL, preferably from about 5 mg/mL to about 40 mg/mL and most preferably from about 20-30 mg/mL is an exemplary starting protein concentration. The protein is generally present in solution. For example, the protein may be present in a pH-buffered solution at a pH from about 4-8, and preferably from about 5-7. Exemplary buffers include histidine, phosphate, Tris, citrate, succinate and other organic acids. The buffer concentration can be from about 1 mM to about 20 mM, or from about 3 mM to about 15 mM, depending, for example, on the buffer and the desired isotonicity of the formulation (e.g. of the reconstituted formulation). The preferred buffer is histidine in that, as demonstrated below, this can have lyoprotective properties. Succinate was shown to be another useful buffer.

**[0065]** The lyoprotectant is added to the pre-lyophilized formulation. In preferred embodiments, the lyoprotectant is a non-reducing sugar such as sucrose or trehalose. The amount of lyoprotectant in the pre-lyophilized formulation is generally such that, upon reconstitution, the resulting formulation will be isotonic. However, hypertonic reconstituted formulations may also be suitable. In addition, the amount of lyoprotectant must not be too low such that an unacceptable amount of degradation/aggregation of the protein occurs upon lyophilization. Where the lyoprotectant is a sugar (such as sucrose or trehalose) and the protein is an antibody, exemplary lyoprotectant concentrations in the pre-lyophilized formulation are from about 10 mM to about 400 mM, and preferably from about 30 mM to about 300 mM, and most preferably from about 50 mM to about 100 mM.

**[0066]** The ratio of protein to lyoprotectant is selected for each protein and lyoprotectant combination. In the case of an antibody as the protein of choice and a sugar (e.g., sucrose or trehalose) as the lyoprotectant for generating an isotonic reconstituted formulation with a high protein concentration, the molar ratio of lyoprotectant to antibody may be from about 100 to about 1500 moles lyoprotectant to 1 mole antibody, and preferably from about

200 to about 1000 moles of lyoprotectant to 1 mole antibody, for example from about 200 to about 600 moles of lyoprotectant to 1 mole antibody.

**[0067]** In preferred embodiments of the invention, it has been found to be desirable to add a surfactant to the pre-lyophilized formulation. Alternatively, or in addition, the surfactant may be added to the lyophilized formulation and/or the reconstituted formulation. Exemplary surfactants include nonionic surfactants such as polysorbates (e.g. polysorbates 20 or 80); poloxamers (e.g. poloxamer 188); Triton; sodium dodecyl sulfate (SDS); sodium laurel sulfate; sodium octyl glycoside; lauryl-, myristyl-, linoleyl-, or stearyl-sulfobetaine; lauryl-, myristyl-, linoleyl- or stearyl-sarcosine; linoleyl-, myristyl-, or cetyl-betaine; lauroamidopropyl-, cocamidopropyl-, linoleamidopropyl-, myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-betaine (e.g. lauroamidopropyl); myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-dimethylamine; sodium methyl cocoyl-, or disodium methyl oleyl-taurate; and the MONAQUAT™ series (Mona Industries, Inc., Paterson, N.J.), polyethyl glycol, polypropyl glycol, and copolymers of ethylene and propylene glycol (e.g. Pluronic, PF68, etc.). The amount of surfactant added is such that it reduces aggregation of the reconstituted protein and minimizes the formation of particulates after reconstitution. For example, the surfactant may be present in the pre-lyophilized formulation in an amount from about 0.001-0.5%, and preferably from about 0.005-0.05%.

**[0068]** In certain embodiments of the invention, a mixture of the lyoprotectant (such as sucrose or trehalose) and a bulking agent (e.g. mannitol or glycine) is used in the preparation of the pre-lyophilization formulation. The bulking agent may allow for the production of a uniform lyophilized cake without excessive pockets therein etc.

**[0069]** Other pharmaceutically acceptable carriers, excipients or stabilizers such as those described in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980) may be included in the pre-lyophilized formulation (and/or the lyophilized formulation and/or the reconstituted formulation) provided that they do not adversely affect the desired characteristics of the formulation. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed and include; additional buffering agents; preservatives; co-solvents; antioxidants including ascorbic acid and methionine; chelating agents such as EDTA; metal complexes (e.g. Zn-protein complexes); biodegradable polymers such as polyesters; and/or salt-forming counterions such as sodium.

**[0070]** The pharmaceutical compositions and formulations described herein are preferably stable. A "stable" formulation/composition is one in which the antibody therein essentially retains its physical and chemical stability and integrity upon storage. Various

analytical techniques for measuring protein stability are available in the art and are reviewed in Peptide and Protein Drug Delivery, 247-301, Vincent Lee Ed., Marcel Dekker, Inc., New York, N.Y., Pubs. (1991) and Jones, A. Adv. Drug Delivery Rev. 10: 29-90 (1993). Stability can be measured at a selected temperature for a selected time period.

**[0071]** The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to, or following, lyophilization and reconstitution. Alternatively, sterility of the entire mixture may be accomplished by autoclaving the ingredients, except for protein, at about 120 °C. for about 30 minutes, for example.

**[0072]** After the protein, lyoprotectant and other optional components are mixed together, the formulation is lyophilized. Many different freeze-dryers are available for this purpose such as Hull50<sup>®</sup> (Hull, USA) or GT20<sup>®</sup> (Leybold-Heraeus, Germany) freeze-dryers. Freeze-drying is accomplished by freezing the formulation and subsequently subliming ice from the frozen content at a temperature suitable for primary drying. Under this condition, the product temperature is below the eutectic point or the collapse temperature of the formulation. Typically, the shelf temperature for the primary drying will range from about -30 to 25 °C (provided the product remains frozen during primary drying) at a suitable pressure, ranging typically from about 50 to 250 mTorr. The formulation, size and type of the container holding the sample (e.g., glass vial) and the volume of liquid will mainly dictate the time required for drying, which can range from a few hours to several days (e.g. 40-60 hours). A secondary drying stage may be carried out at about 0-40 °C., depending primarily on the type and size of container and the type of protein employed. However, it was found herein that a secondary drying step may not be necessary. For example, the shelf temperature throughout the entire water removal phase of lyophilization may be from about 15-30 °C. (e.g., about 20 °C.). The time and pressure required for secondary drying will be that which produces a suitable lyophilized cake, dependent, e.g., on the temperature and other parameters. The secondary drying time is dictated by the desired residual moisture level in the product and typically takes at least about 5 hours (e.g. 10-15 hours). The pressure may be the same as that employed during the primary drying step. Freeze-drying conditions can be varied depending on the formulation and vial size.

**[0073]** In some instances, it may be desirable to lyophilize the protein formulation in the container in which reconstitution of the protein is to be carried out in order to avoid a transfer step. The container in this instance may, for example, be a 3, 5, 10, 20, 50 or 100 cc

vial. As a general proposition, lyophilization will result in a lyophilized formulation in which the moisture content thereof is less than about 5%, and preferably less than about 3%.

**[0074]** At the desired stage, typically when it is time to administer the protein to the patient, the lyophilized formulation may be reconstituted with a diluent such that the protein concentration in the reconstituted formulation is at least 50 mg/mL, for example from about 50 mg/mL to about 400 mg/mL, more preferably from about 80 mg/mL to about 300 mg/mL, and most preferably from about 90 mg/mL to about 150 mg/mL. Such high protein concentrations in the reconstituted formulation are considered to be particularly useful where subcutaneous delivery of the reconstituted formulation is intended. However, for other routes of administration, such as intravenous administration, lower concentrations of the protein in the reconstituted formulation may be desired (for example from about 5-50 mg/mL, or from about 10-40 mg/mL protein in the reconstituted formulation). In certain embodiments, the protein concentration in the reconstituted formulation is significantly higher than that in the pre-lyophilized formulation. For example, the protein concentration in the reconstituted formulation may be about 2-40 times, preferably 3-10 times and most preferably 3-6 times (e.g. at least three fold or at least four fold) that of the pre-lyophilized formulation.

**[0075]** Reconstitution generally takes place at a temperature of about 25 °C. to ensure complete hydration, although other temperatures may be employed as desired. The time required for reconstitution will depend, e.g., on the type of diluent, amount of excipient(s) and protein. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (e.g. phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution. The diluent optionally contains a preservative. Exemplary preservatives have been described above, with aromatic alcohols such as benzyl or phenol alcohol being the preferred preservatives. The amount of preservative employed is determined by assessing different preservative concentrations for compatibility with the protein and preservative efficacy testing. For example, if the preservative is an aromatic alcohol (such as benzyl alcohol), it can be present in an amount from about 0.1-2.0% and preferably from about 0.5-1.5%, but most preferably about 1.0-1.2%. Preferably, the reconstituted formulation has less than 6000 particles per vial which are >10 µm in size.

### **Therapeutic Applications**

**[0076]** The glycoengineered antibodies described herein may be used for treating a patient having a cancer. The method of the treatment comprises administering to the patient an effective amount of a glycoengineered antibody or a pharmaceutical composition described herein. Examples of the cancers include, but are not limited to cancers associated with and/or

expressing Globo series antigens, including, but not limited to, Globo H, SSEA-4, SSEA-3; cancers associated with and/or expressing Her-2; cancers associated with and/or expressing EGFR receptor.

**[0077]** In certain embodiments, the cancer is a breast cancer.

**[0078]** Further, the glycoengineered antibodies described herein may be used for treating a patient having an autoimmune or inflammatory disease. The method of the treatment comprises administering to the patient an effective amount of a glycoengineered antibody or a pharmaceutical composition described herein. Examples of the autoimmune or inflammatory disease include, but are not limited to, rheumatoid arthritis, juvenile rheumatoid arthritis, systemic lupus erythematosus (SLE), Wegener's disease, inflammatory bowel disease, idiopathic thrombocytopenic purpura (ITP), thrombotic thrombocytopenic purpura (TTP), autoimmune thrombocytopenia, multiple sclerosis, psoriasis, IgA nephropathy, IgM polyneuropathies, myasthenia gravis, vasculitis, diabetes mellitus, Reynaud's syndrome, Crohn's disease, ulcerative colitis, gastritis, Hashimoto's thyroiditis, ankylosing spondylitis, hepatitis C-associated cryoglobulinemic vasculitis, chronic focal encephalitis, bullous pemphigoid, hemophilia A, membranoproliferative glomerulonephritis, adult and juvenile dermatomyositis, adult polymyositis, chronic urticaria, primary biliary cirrhosis, neuromyelitis optica, Graves' dysthyroid disease, bullous pemphigoid, membranoproliferative glomerulonephritis, Churg-Strauss syndrome, asthma, psoriatic arthritis, dermatitis, respiratory distress syndrome, meningitis, encephalitis, uveitis, eczema, atherosclerosis, leukocyte adhesion deficiency, juvenile onset diabetes, Reiter's disease, Behcet's disease, hemolytic anemia, atopic dermatitis, Wegener's granulomatosis, Omenn's syndrome, chronic renal failure, acute infectious mononucleosis, HIV and herpes-associated disease, systemic sclerosis, Sjogren's syndrome and glomerulonephritis, dermatomyositis, ANCA, aplastic anemia, autoimmune hemolytic anemia (AIHA), factor VIII deficiency, hemophilia A, autoimmune neutropenia, Castleman's syndrome, Goodpasture's syndrome, solid organ transplant rejection, graft versus host disease (GVHD), autoimmune hepatitis, lymphoid interstitial pneumonitis (HIV), bronchiolitis obliterans (non-transplant), Guillain-Barre Syndrome, large vessel vasculitis, giant cell (Takayasu's) arteritis, medium vessel vasculitis, Kawasaki's Disease, and polyarteritis nodosa.

**[0079]** In certain embodiments, the autoimmune or inflammatory disease is rheumatoid arthritis.

[0080] In these treatment methods, the primary glycoengineered antibody can be administered alone or in conjunction with a second therapeutic agent such as a second antibody, or a chemotherapeutic agent or an immunosuppressive agent.

### EXAMPLES

[0081] Embodiments of the invention will be further illustrated with the following specific examples. One skilled in the art would appreciate that these specific examples are for illustration only and that other modifications and variations are possible without departing from the scope of the invention. For example, the enzyme variants of the invention may be used to glycoengineer any glycoproteins or glycopeptides, including antibodies. The specific examples described herein use anti-CD20 antibodies. However, one skilled in the art would appreciate that other glycoproteins or antibodies may also be used in a similar manner.

### Materials

[0082] Monoclonal anti-Globo H antibody, OBI-888 was produced according to our previously procedure disclosed in PCT patent publications (WO2015157629A2 and WO2017062792A1). Monoclonal anti-SSEA4 antibody, OBI-898 was produced according to our previously procedure disclosed in PCT patent publication (WO2017172990A1). The commercial antibodies Herceptin (trastuzumab), Perjeta (pertuzumab), Erbitux (cetuximab), Rituxan (rituximab), Vectibix (panitumumab), Humira (adalimumab), Keytruda (pembrolizumab) and Bavencio (avelumab). were purchased from:

|       | Herceptin         | Perjeta  | Erbitux     | Rituxan  | Vectibix | Humira    | Keytruda    | Bavencio           |
|-------|-------------------|----------|-------------|----------|----------|-----------|-------------|--------------------|
| Brand | Roche             | Roche    | MerckSerono | Roche    | Amgen    | AbbVie    | MSD Ireland | Merck              |
| Lot   | N7208B06<br>B3066 | H0239B09 | 245011      | H0229B13 | 1080265  | 83347XH04 | 7302614A13  | 04150132<br>280583 |

[0083] Biantennary glycan, Sialylated complex type N-glycan (NSCT), was purchase from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan, D4065) and NSCT-oxazoline were synthesized according to previously reported (Noguchi, M. *et al.* (2012) *Helvetica chimica acta* 95: 1928–1936). Other glycans (M3, G0, and G2) and Bf- $\alpha$ -fucosidase was produced according previous papers (Tsai, T. I. *et al.* (2017) *ACS Chem. Biol.* 12: 63–72; Fairbanks, A. J. (2013) *Pure Appl. Chem.* 85, 1847-1863).

**Example 1: Cloning, overexpression and purification of EndoSd-D232M and EndoSz-D234M and mutants**

**[0084]** The genes of EndoSd and EndoSz from *Streptococcus dysgalactiae subsp. Dysgalactiae* (ANI26082.1) and *Streptococcus equi subsp. Zooepidemicus Sz105* (KIS14581.1) were used for this study. The signal peptides were deleted in both enzymes at N-terminal. To enhance transglycosylation activity we aligned the EndoSd and EndoSz protein sequence to EndoS-D233Q (Huang, W. *et al.* (2012) *J. Am. Chem. Soc.* 134: 12308–12318) and found the relative position is D232 and D234 for EndoSd and EndoSz, respectively. We decided to mutate the relative position D to M. Therefore, the gene encoding amino acids 20-1067 of EndoSd-D232M and 20-1011 of EndoSz-D234M were synthesized and sub-clone into pGEX-4T-1 with 5'-BamHI and 3'-XhoI restriction sites. For the purification purpose, we inserted additional six histidine at the C-terminal of EndoSd-D232M and EndoSz-D234M for affinity Ni-NTA column. Other mutants used in this investigation were generated by site-directed mutagenesis. Related primers were designed base on mutated sites. Taking EndoSd-D232M and EndoSz-D234M as template vectors, the mutated vectors were amplified by Pfu DNA polymerase (Protech). Then, the template vectors (methylated DNA) were digested by DpnI (Promega) for 2 hours (37°C). The mutated vectors were transformed to the DH5 $\alpha$  competent cell for selection. All mutants were confirmed by DNA sequencing.

**[0085]** All vectors were transformed into BL21 (DE3) and cultured at 37 °C in TB medium containing ampicillin antibiotic (50  $\mu$ g/mL). The proteins were induced by 0.2 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) while the cell density OD600 reached 0.6. After 5 hours, the cells were harvested at 25 °C by centrifugation (BACKMAN/JLA-8.1, 9000 g) for 15 minutes. The cell pellet was resuspension with wash buffer containing 50 mM MOPS pH 7.0, 300 mM NaCl and 10 mM imidazole (100 mL buffer/1L cell pellet) for the homogenizer (NanoLyzer N-10) to break the cell. After 60 min/12,000 g (BACKMAN/JA-10) centrifugation at 4 °C and discard pellet, the supernatant was mixed with Ni-NTA resin (Roche) and gentle rocked overnight at 4 °C for protein binding completely. The resin was loaded onto open column and washed non-bound protein with wash buffer until the concentration of non-bound protein was less than 1 mg/mL (defined by Bradford assay, Thermo). The bound protein was eluted with elute buffer containing 50 mM MOPS pH 7.0, 300 mM NaCl and 250 mM imidazole. The eluted fraction was dialysis to a storage buffer containing 50 mM MOPS pH 6.7 and concentrated using TFF (Millipore lab scale) by 30kDa cutoff cassette. The final samples were assayed by SDS-PAGE and Bradford for detecting MW and concentrates, respectively.

**Example 2: Deglycosylation of OBI-888 by EndoS-WT and Bf- $\alpha$ -fucosidase to generate mAb-GlcNAc and mAb-GlcNAc(F)**

[0086] The OBI-888 and Herceptin monoclonal antibody (10 mg) were incubated with EndoS (10  $\mu$ g) in a 25 mM sodium citrate buffer (pH 6.5) and 100mM NaCl at 37 °C for 4 h. The complete cleavage of Fc N-glycans was analyzed by 4-12 % gradient SDS-PAGE.

[0087] The N-glycans of OBI-888 (10 mg) was digesting by incubation with EndoS-WT (10  $\mu$ g) and Bf- $\alpha$ -fucosidase (10 mg) in the Tris-HCl buffer (pH 7.4) at 37 °C for 16 hours to generate OBI-888-GlcNAc. The commercial antibodies Herceptin (trastuzumab), Perjeta (pertuzumab), Erbitux (cetuximab), Rituxan (rituximab), Vectibix (panitumumab), Humira (adalimumab), Keytruda (pembrolizumab) and Bavencio (avelumab) (10 mg) used the same procedure as OBI-888 except temperature at 30 °C. The complete cleavage of Fc N-glycans was analyzed by 4-12 % gradient SDS-PAGE. Fucosylated mAb-GlcNAc (mAbs-GlcNAc-F) were produced by only EndoSz wild type in similar condition with 4 hours incubation time.

**Example 3: Transglycosylation of glycans to mAb-GlcNAc and mAb-GlcNAc-F**

[0088] In general procedures, mAb-GlcNAc/mAb-GlcNAc-F (5 mg) was incubated with glycan-oxa with molar ratio 1:20 and 1:150 (mAb-GlcNAc : NSCT-oxa) by EndoSz-D234M (167  $\mu$ g) or EndoSd-D232M (1002  $\mu$ g), respectively, at 30°C for 20 minutes in a MOPS buffer (50 mM, pH 6.7), to final volume 500  $\mu$ L. There were some slight modifications according to experiment purposes and designs (see result section). HPLC was employed to monitor the transglycosylation efficiency.

**Example 4: Purification of deglycosylated and homogeneous mAb**

[0089] The reaction mixture was applied to a HiTrap Protein-A HP (5 mL, GE) prepack column which pre-equilibrated with PBS buffer. The non-bound contaminations were washed by two steps pH gradient, PBS (pH 7.4) buffer and glycine-HCl (pH 5.0) buffer, with five times of column volume in each step. Sodium citrate (pH 3.0) was employed to elute bound antibody. The eluted fractions were immediately neutralized with Tris-HCl buffer (1M, pH 9.0) to pH 7.4 and dialyzed to the storage buffer containing 50 mM MOPS (pH 6.7) for mAb-GlcNAc(F) and 5mM Histidine and 150 mM Nacl for mAb-G2S2, respectively, with 30 kDa cutoff dialysis cassette (Thermo) overnight at 4 °C. All samples were concentrated by Amicon centrifugation membrane (30 kDa cutoff, Millipore) and storage at 4°C [mAb-GlcNAc(F)] or -80°C [mAb-G2S2(F)].

**Example 5: LC/MS/MS of glycopeptide analysis**

[0090] Samples were first processed for buffer exchange into ddH<sub>2</sub>O by 10 kDa cut-off Amicon Ultra-0.5 device. Samples were denatured in 0.1% RapiGest SF solution/50 mM triethylammonium bicarbonate (TEABC), reduced with 5mM Dithiothreitol (DTT) at 60 °C for 30 minutes, then alkylated using 15mM iodoacetamide in dark at room temperature for 30 minutes. The resulting samples were subjected to in-solution digestion with trypsin (trypsin: sample protein=1:30) in 50 mM TEABC at 37 °C overnight. After digestion, the samples were acidified with 0.5% trifluoroacetic acid (TFA) (v/v) and incubated at 37°C for 45 minutes. The acid treated samples were centrifuged at 4°C, 14000 rpm for 30 minutes to precipitate the hydrolytic RapiGest SF by-product. The samples were analyzed with Thermo Q-Exactive mass spectrometer (Thermo Scientific) coupled with Ultimate 3000 RSLC system (Dionex). The LC separation was performed using the C18 column (Acclaim PepMap RSLC, 75 μm x 150 mm, Thermo) with mobile phase A: 0.1% FA (Formic acid) and B: 95% ACN (acetonitrile) / 0.1% FA and Table 2 listed the analysis solvent gradient.

[0091] Table 2. Solvent gradient of LC

| Time (min) | A% | B% | Flow (μL/min) |
|------------|----|----|---------------|
| 0          | 99 | 1  | 0.25          |
| 5.5        | 99 | 1  | 0.25          |
| 45         | 75 | 25 | 0.25          |
| 48         | 40 | 60 | 0.25          |
| 50         | 20 | 80 | 0.25          |
| 60         | 20 | 80 | 0.25          |
| 65         | 99 | 1  | 0.25          |
| 70         | 99 | 1  | 0.25          |

[0092] Full MS scan was performed with m/z range of 300 to 2000 and the ten most intense ions from MS scan were selected for MS/MS scans.

**Example 6: Enzymatic conjugation assay by HPLC**

[0093] The glycosynthase activity was analyzed by HPLC (Waters e2695) using 2.1 x 150 mm UPLC Glycoprotein Amide Column (Waters) under two different buffers (Buffer A,

ddH<sub>2</sub>O/0.3 % v/v HFIP (1,1,1,3,3,3-Hexafluoro-2-propanol), 0.1 % v/v TFA; Buffer B, ACN, 0.3 % v/v HFIP, 0.1 % v/v TFA) with the gradient as Table 3.

**[0094]** Table 3. Solvent gradient of HPLC

| Time | Flow (mL/min) | A (%) | B (%) |
|------|---------------|-------|-------|
| 0.00 | 0.20          | 15    | 85    |
| 0.50 | 0.20          | 15    | 85    |
| 1.00 | 0.20          | 33    | 67    |
| 17.0 | 0.20          | 38.6  | 61.4  |
| 20.0 | 0.20          | 38.6  | 61.4  |
| 21.0 | 0.20          | 15    | 85    |
| 30.0 | 0.20          | 15    | 85    |

**[0095]** Before experiment the column was washed by 50% ACN and 50% of ddH<sub>2</sub>O for 30 minutes and equilibrated with 15% buffer A and 85 % buffer B until the system pressure was stable. After ensuring the baseline stability by injection water blank, the process was started by 2 µL sample injection. In the process duration (29 mins), the flow rate was 0.2 mL/min under the column temperature of 65°C and sample tray temperature of 5°C.

#### **Example 7: ADCC assay of the engineered antibody**

**[0096]** The ADCC activity was analyzed by ADCC reporter bioassay complete kit (Promega, G7015) using luciferase reporter cells. The related cell lines MCF7W (OBI-888), SKBR-3 (Herceptin, Perjeta), BxPC3 (Erbix) and Raji (Rituxan) were selected for the analysis. Cell lines shared with the same procedures. Target cells were seeded at 96-well cell culture plate and incubated overnight at 37°C in a humidified 5% CO<sub>2</sub> incubator. The culture medium was replaced with serial dilution of homogeneous antibody and the corresponding antibody standard in triplicate. In each well, ADCC bioassay effector cells were added. Ratio of effector cell to target cell was 3:1. We performed induction for 6 hours and then added of Bio-Glo luciferase assay buffer. After 15 minutes, luminescence (RLU, relative light unit) was determined using microplate reader (SpectraMax L, Molecular Devices, Sunnyvale, CA). The fold change of luminescence induction was calculated by the ratio of relative light unit (RLU) (induced) to RLU (no antibody control). EC<sub>50</sub> was determined by plotting x (concentration in µg/mL) –y (the induction of fold change) and fitting the data in a 4PL nonlinear regression

model by PRISM 6 Software. Relative potency was estimated by parallel line analysis using Gen5 Microplate Reader and Imager Software (BioTek Instruments).

### **Example 8: Cleavage and Conjugation of Herceptin**

**[0097]** Antibody produced by CHO cell always contains heterogeneous glycans on the N297 position of Fc. To generate homogeneous mAbs and enhance ADCC, enzymatic modification of glycan cleavage and transglycosylation are essential steps in homogeneous platform (Fig. 1). For the glycan cleavage, it has reported that EndoSd-WT hydrolyzed biantennary glycans but it is not a general chitinase (Shadnezhad, A. *et al.* (2016) *Future Microbiol* 11: 721–736). To illustrate the ability of EndoSz-WT, it was used to hydrolyze native Herceptin and detected the N-glycan profile. The result showed EndoSz-WT could hydrolyzed biantennary hybrid and high mannose glycans. It was generated >99% Herceptin with only one N-acetylglucosamine (Herceptin-GlcNAc) on N297 by EndoSz-WT and  $\alpha$ -fucosidase enzyme (Fig. 2). However, for the mAb containing glycosylated Fab, we combined additional enzyme, EndoH or EndoM (Shadnezhad, A. *et al.* (2016) *Future Microbiol* 11: 721–736; Kadowaki, S. *et al.* (1990) *Agric. Biol. Chem.* 54: 97–106), in the cleavage step for the glycan removal completely.

**[0098]** ENGases also have glycan conjugation function with proper mutations (Huang, W. *et al.* (2012) *J. Am. Chem. Soc.* 134: 12308–12318; Li., T., Tong, *et al.* (2016) *J. Biol. Chem.* 291: 16508–16518). We have established the HPLC method with amide column for precisely monitoring the transglycosylation process. As the example showed in Fig. 3, native Herceptin had retention time at 12.5 minute. After EndoSz-WT and  $\alpha$ -fucosidase cleavage, the retention time of Herceptin with one GlcNAc (Herceptin-GlcNAc) was shifted to 11.4 minute and had the calculated molecular mass MW=145,572 Da by intact mass analysis. In the transglycosylation step of oxazoline sialylated complex type N-glycan (NSCT-oxa) to Herceptin-GlcNAc (demonstrated by EndoSz-D234M), it is interesting to note that the column could clearly identify hemi-glycosylated Herceptin (Herceptin-1N-G2S2) and fully-glycosylated Herceptin (Herceptin-2N-G2S2) that could not be distinguished in the SDS-PAGE. Herceptin-1N-G2S2 was found in the retention time at 12.6 minute with MW= 147,574 Da, whereas Herceptin-2N-G2S2 had retention time at 13.9 minute with M=149,576 Da. The HPLC method is able to apply to all mAbs. Therefore, we used HPLC assay for further investigation.

**Example 9: Transglycosylation investigation of EndoSz-D234M and EndoSd-D232M**

**[0099]** Transglycosylation is the most important step in homogeneous platform that decides the quality of homogeneous mAbs. According to previous report (Li, T., Tong, *et al.* (2016) *J. Biol. Chem.* 291: 16508–16518), EndoS2 employed D184M mutation had high transglycosylation activity. We have first generated EndoSz-D234M and EndoSd-D232M for the transglycosylation investigation by multiple sequence alignment (Fig. 4). In general, higher sugar ratio would generate higher conjugation efficiency. However, in industry used, decreasing sugar amount to save cost and processing with a reasonable time frame were the goals for optimizing the process. We used Herceptin-GlcNAc and NSCT-oxa as an investigate model to evaluate the transglycosylation activity of EndoSz-D234M and EndoSd-D232M within 60 minutes and expected the formation of >90% Herceptin-2N-G2S2.

**[00100]** Starting with NSCT-oxa/Herceptin molar ratio of 40:1, >90% Herceptin-2N-G2S2 was obtained with EndoSz-D234M. Decrease the amount of NSCT-oxa/Herceptin molar ratio of 30:1, > 90% Herceptin-2N-G2S2 reached at 10 minutes. With 20:1 of NSCT-oxa/Herceptin ratio, Herceptin-2N-G2S2 reached to 89.31% at 5 minutes, reached to 91.85% at 10 minutes and stayed until 20 minutes before deglycosylating (Fig. 5A). With 10:1 of NSCT-oxa/Herceptin (molar ratio), 62.61% of Herceptin-2N-G2S2 were obtained at 5 minutes, 61.48% at 10 minutes and deglycosylation started. To determine extremity of NSCT-oxa usage, increase amount of NSCT-oxa/Herceptin to 15:1 (molar ratio). 80.8% of Herceptin-2N-G2S2 was attained at 5 minutes, and deglycosylation started at 10 minutes. Therefore, the final condition of transglycosylation with EndoSz-D234M enzyme was NSCT-oxa : antibody = 20:1 (molar ratio) with 20 minutes reaction time.

**[00101]** The time dependent graph of EndoSz-D234M (Fig. 5A) clearly illustrated the enzyme behavior. It is an efficiency enzyme that quickly bound to Fc and conjugated glycan to N297 position of Fc as we saw that within five minutes Herceptin-GlcNAc decreased almost to 0% and most of fully glycosylated antibody was found. However, the high efficiency transglycosylation enzyme also contributes to hydrolysis activity. In all trails, deglycosylation occurred while the reaction reached the peak efficiency of transglycosylation and stay for a period of time (20 minutes), which indicated the importance of controlling the time frame in the process. Notably, the data showed decreasing percentage of Herceptin-2N-G2S2 accompanied by increasing major percentage of Herceptin-1N-G2S2 and minor percentage of Herceptin-GlcNAc, implying the enzyme has priority to select the targets in the hydrolysis reaction.

**[00102]** In contrast, applying the final transglycosylation condition of EndoSz-D234M onto EndoSd-D232M enzyme showed inconsistent results. Only 46.06% Herceptin-2N-G2S2 was generated by EndoSd-D232M with molar ratio 20:1 (NSCT-oxa/antibody), which demonstrated a better glycosynthase activity of EndoSz-D234M. In order to obtain higher fully glycosylated antibody, increasing amount of EndoSd-D232M and NSCT-oxa were the strategies to enhance transglycosylation efficiency. By increasing enzyme amount five times, it was too poor to meet our expected efficiency (>90%) by only 60% Herceptin-2N-G2S2 produced. Therefore, we tried to increase NSCT-oxa amount to 80:1 (molar ratio) and give an unstable result with the range of 80-90% Herceptin-2N-G2S2. Finally, 150:1 (molar ratio) of NSCT-oxa gave a stable and repeatable data. The time dependent graph (Fig. 5B) showed that the EndoSd-D232M slowly transferred NSCT-oxa to N297 (Fc region) despite larger amount of substrate was used. In 5 minutes, the Herceptin-1N-G2S2 (~50%) has larger amount than Herceptin-2N-G2S2 (~40%). Herceptin-2N-G2S2 reached to 80% at 10 minutes and 94% at 20 minutes. The deglycosylation was not found within 60 minutes.

**[00103]** Besides using Herceptin-GlcNAc as acceptor, we also studied transglycosylation activity of fucosylated Herceptin-GlcNAc (Herceptin-GlcNAc-F) that potentially applies in Antibody-Drug-Conjugation (ADC). Using the best transglycosylation conditions described previously, it was obtained 94.29% and 94.75% Herceptin-2N-G2S2F by EndoSz-D234M and EndoSd-D232M, respectively (Table 4). It has demonstrated that two enzymes have transglycosylation activity on fucosylated substrate.

**[00104]** Table 4. The transglycosylation results of EndoSz-D234M and EndoSd-D232M in different acceptors.

| Enzyme       | Acceptor           | GlcNAc(F) | 1N-G2S2 | 2N-G2S2 |
|--------------|--------------------|-----------|---------|---------|
| EndoSz-D234M | Herceptin-GlcNAc   | 3.35%     | 4.8%    | 91.85%  |
|              | Herceptin-GlcNAc-F | 1.72%     | 4.00%   | 94.29%  |
| EndoSd-D232M | Herceptin-GlcNAc   | 0%        | 2.11%   | 97.88 % |
|              | Herceptin-GlcNAc-F | 0%        | 3.17%   | 94.75%  |

**[00105]** In conclusion, EndoSz-D234M has a better transglycosylation activity than EndoSd-D232M. EndoSz-D234M stable produced >90% Herceptin-2N-G2S2 (Herceptin-2N-G2S2F) with only 20:1 molar ratio (NSCT-oxa : antibody), whereas EndoSd-D232M needs 7.5 folds of substrate

**Example 10: Transglycosylation investigation of EndoSz and EndoSd mutates**

**[00106]** The previous reports that several mutant sites, such as EndoS-D233Q and EndoS2-D184M could increase transglycosylation activity for glycan (Huang, W. *et al.* (2012) *J. Am. Chem. Soc.* 134: 12308–12318; Li., T., Tong, *et al.* (2016) *J. Biol. Chem.* 291: 16508–16518). Recently, Shivatare *et al.* reported that the mutation of EndoS2-T138Q increases better activity than EndoS2-D184M (Shivatare, S. S. *et al.* (2018) *Chem. Commun.* 54, 6161-6164). According to multiple sequence alignment (Fig. 4), the equivalent positions T183, D232, D234, D280, S281 and T282 of EndoSz, and T181, D230, D232, D278, S279 and T280 of EndoSd were selected as targets for site-directed mutagenesis investigation, in which EndoSz D234 and EndoSd D232 sites were generated by several different types, including positive/negative charge and polar/non-polar. The transglycosylation activity was assayed using the best conditions described previous.

**[00107]** The activity assay results of EndoSz mutants (Fig. 6A) showed that EndoSz-D234M has highest activity (set to 100%) and EndoSz-D234Q (99.9%), EndoSz-D234S (98.8%) and D234F (98.6%) have competitive high activities. In contrast, EndoSz-D234R (4.9%) and EndoSz-D234H (4.5%) have low activities. EndoSz wild type also has slight transglycosylation activity (24.3%). Beside the D234 position, EndoSz-T183Q (89.1%), EndoSz-D232Q (31.4%), EndoSz-D280Q (34.0%), EndoSz-S281Q (12.9%) and EndoSz-T282Q (16.3%) had no significant increasing transglycosylation activity compared to EndoSz-D234M.

**[00108]** In EndoSd mutants (Fig. 6B), EndoSd-D232M (set to 100%), EndoSd-D232S (100.8%) and EndoSd-D278Q (108.0%) have equally high transglycosylation activity, whereas EndoSd-D232R (9.2%) and EndoSd-D232H (8.1%) have low activities. The EndoSd wild type had relatively high transglycosylation activity (84%).

**[00109]** In conclusion, EndoSz-D234M and EndoSd-D278Q were shown to exhibit relative better transglycosylation activity on Herceptin-GlcNAc to produce homogeneous Herceptin bearing with NSCT-oxa at the Fc region.

**Example 11: Conjugation investigation of EndoSz-D234M on various sugars**

**[00110]** Homogeneous platform was designed to conjugate various glycans on Fc region of Herceptin. Glycans, M3, G0 and G2, were used for the investigation by better effective enzyme, EndoSz-D234M. The results showed all of the glycans were successfully conjugated

onto Fc region with 20:1 molar ratio (NSCT-oxa : antibody). G0 and G2 except M3 reached to >90% fully glycosylated Herceptin (Table 5).

[00111] Table 5. The transglycosylation results of EndoSz-D234M in different acceptor with different glycans.

| Acceptor           | Glycan type | GlcNAc(F) | 1N-Glycan | 2N-Glycan |
|--------------------|-------------|-----------|-----------|-----------|
| Herceptin-GlcNAc   | M3 (40eq)   | 1.11%     | 6.51%     | 92.37%    |
|                    | G0          | 0.91%     | 2.58%     | 96.50%    |
|                    | G2          | 0.61%     | 4.52%     | 94.87%    |
| Herceptin-GlcNAc-F | M3F         | 0.62%     | 4.95%     | 94.44%    |
|                    | G0F         | 0.00%     | 2.45%     | 97.55%    |
|                    | G2F         | 0.21%     | 2.64%     | 97.14%    |

[00112] M3 was able to obtain 78% fully glycosylated Herceptin at 5 minutes and deglycosylation started. To optimize the conjugation rate, increasing M3 to 30:1 (molar ratio) to attain a result of 86.3% at minute 10 before deglycosylation, and increasing to 40:1 (molar ratio) for 90% of fully glycosylated Herceptin attempt. Final condition of conjugating with M3 was 40:1 with 10 minutes reaction time to obtain 92.37% fully glycosylated Herceptin. Homogeneous platform not only can be applied on Herceptin-GlcNAc but also applied on Herceptin-GlcNAc-F to conjugating various glycans on Fc region and with results of above 90% conjugation efficiency.

#### Example 12: Transglycosylation on various antibodies

[00113] Homogeneous platform is a powerful process to establish homogeneous mAbs. Several other mAbs were selected for the conjugation investigation by EndoSz-D234M, including OBI-888, Perjeta, Erbitux, Rituxan, OBI-898, Vectibix, Humira, Keytruda, Bavencio. With the condition of 20:1 (molar ratio) of NSCT-oxa to antibody, the results demonstrated the effectiveness of homogeneous platform (Fig. 7). The percentage of fully glycosylated mAbs are OBI-888-G2S2: 87.57%, Perjeta-G2S2: 92.49%, Erbitux-G2S2: 87.92%, Rituxan-G2S2: 97.57%, OBI-898-G2S2: 89.73%, Vectibix-G2S2: 86.12%, Humira-G2S2: 93.68%, Keytruda-G2S2: 75.81% and Bavencio-G2S2: 90.73%.

[00114] To evaluate the effect of homogeneous glycan on different mAbs, we have selected five antibodies for the ADCC bioassay (Fig. 7B). It is obviously that the EC50 of homogeneous mAbs were increased by comparing with original mAbs. Especially OBI-888 increased 26 folds had the best ADCC improvement.

[00115] The present invention discloses selected glycosynthase variants that show excellent transglycosylation activities with a broad range of N-glycans, including high mannose, hybrid and complex types.

[00116] In preferred embodiments, N-glycans of high mannose, hybrid and complex types are in an active oxazoline form.

[00117] In some embodiments, the high mannose type N-glycans described herein are selected from group consisting of Man<sub>3</sub>GlcNAc, Man<sub>5</sub>GlcNAc, Man<sub>6</sub>GlcNAc, Man<sub>7</sub>GlcNAc, Man<sub>8</sub>GlcNAc, and Man<sub>9</sub>GlcNAc. In preferred embodiments, the high mannose type N-glycan is Man<sub>5</sub>GlcNAc.

[00118] In some embodiments, the hybrid type N-glycans described herein comprise at least one  $\alpha$ -2,6- or  $\alpha$ -2,3 terminal sialic acid on the alpha-1,3 arm, wherein the alpha-1,6 arm contains the trimannose residues.

[00119] In some embodiments, the hybrid type N-glycans described herein comprise at least one terminal galactose on the alpha-1,3 arm, wherein the alpha-1,6 arm contains the trimannose residues.

[00120] In some embodiments, the hybrid type N-glycans described herein comprise at least one terminal GlcNAc on the alpha-1,3 arm, wherein the alpha-1,6 arm contains the trimannose residues.

[00121] In some embodiments, the complex type glycans are of bi-, tri- and tetra-antennary complex types.

[00122] In some embodiments, the bi-antennary complex type N-glycans described herein comprise at least one  $\alpha$ -2,6 or  $\alpha$ -2,3 terminal sialic acid. In preferred embodiments, the N-glycans comprise two  $\alpha$ -2,6 and/or  $\alpha$ -2,3 terminal sialic acids.

[00123] In some embodiments, the bi-antennary complex type N-glycans described herein comprise at least one terminal galactose or GlcNAc. In preferred embodiments, the N-glycans comprise two terminal galactose and/or GlcNAc.

[00124] In some embodiments, the bi-antennary complex type N-glycans described herein comprise at least one alpha-1,2-fucose. In preferred embodiments, the N-glycans comprise two alpha-1,2-fucoses.

[00125] In some embodiments, the bi-antennary complex type N-glycans described herein comprise at least one alpha-1,3-fucose. In preferred embodiments, the N-glycans comprise two alpha-1,3-fucose.

[00126] In some embodiments, the bi-antennary complex type N-glycans described herein comprise bisecting GlcNAc.

**[00127]** In some embodiments, the bi-antennary complex type N-glycans described herein comprise at least one LacNAc repeat unit. In preferred embodiments, the N-glycans comprise two LacNAc repeat units.

**[00128]** In some embodiments, the tri-antennary complex type N-glycans described herein comprise at least one  $\alpha$ -2,6 or  $\alpha$ -2,3 terminal sialic acid. In preferred embodiments, the N-glycans comprise three  $\alpha$ -2-6 and/or  $\alpha$ -2,3 terminal sialic acids.

**[00129]** In some embodiments, the tri-antennary complex type N-glycans described herein comprise at least one terminal galactose or GlcNAc. In preferred embodiments, the N-glycans comprise three terminal galactose and/or GlcNAc.

**[00130]** In some embodiments, the complex type glycans are of bi-, and triantennary complex types comprising asymmetric antennae on either the alpha-1,3 or alpha-1,6 arm.

**[00131]** In some embodiments, the hybrid and bi-, and triantennary complex type N-glycans described herein comprise  $\alpha$ -2,6 or  $\alpha$ -2,3 terminal sialic acid. In other embodiments, the hybrid and bi-, and triantennary complex type N-glycan comprises  $\alpha$ -2,6 terminal sialic acid.

**[00132]** While the invention has been described with respect to a limited number of embodiments, those skilled in the art, having benefit of this disclosure, will appreciate that other embodiments can be devised which do not depart from the scope of the invention as disclosed herein. Accordingly, the scope of the invention should be limited only by the attached claims.

**[00133]** Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skills in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications and patents specifically mentioned herein are incorporated by reference for all purposes including describing and disclosing the chemicals, cell lines, vectors, animals, instruments, statistical analysis and methodologies which are reported in the publications which might be used in connection with the invention. All references cited in this specification are to be taken as indicative of the level of skill in the art. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

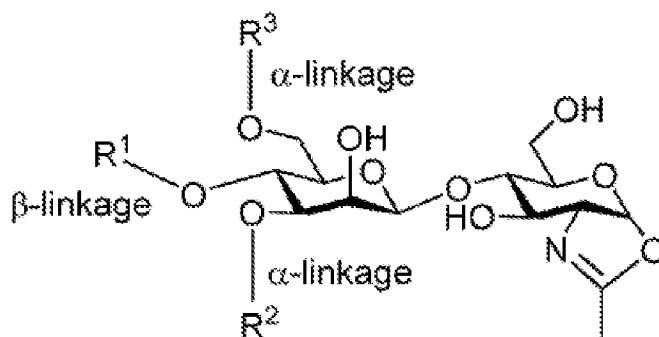
**[00134]** Before the present materials and methods are described, it is understood that this invention is not limited to the particular methodology, protocols, materials, and reagents described, as these may vary. It is also to be understood that the terminology used herein is for

the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

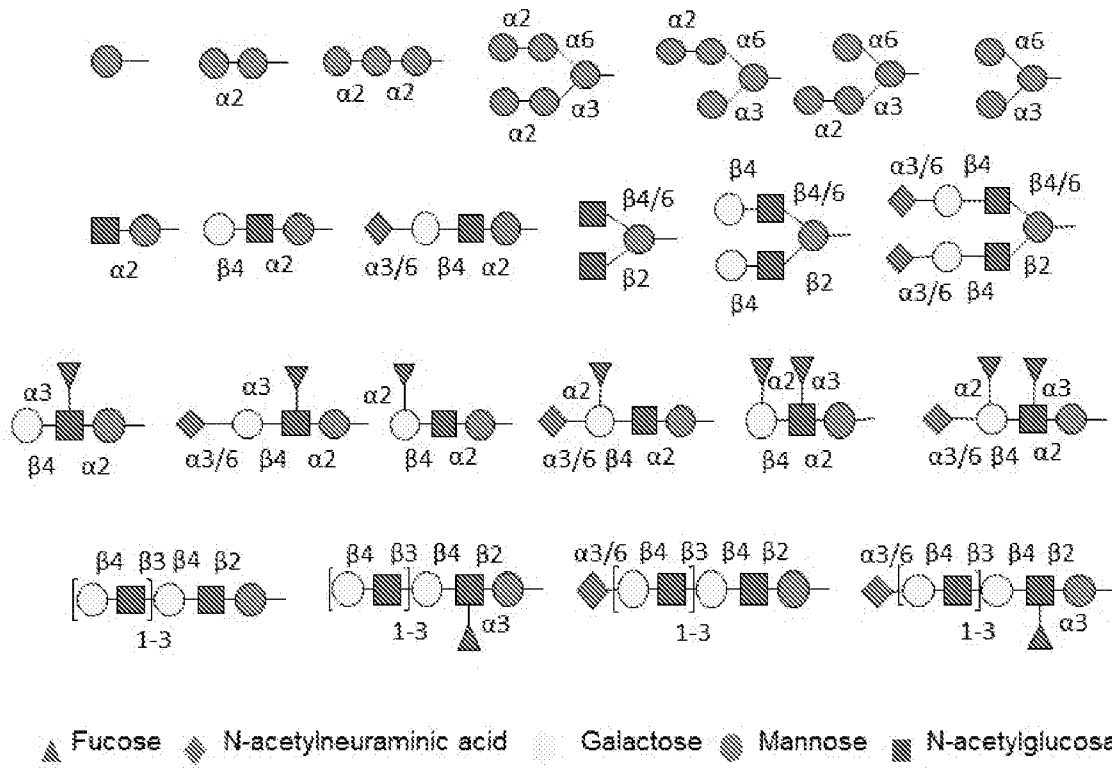
## CLAMS

What is claimed is:

1. A mutant of glycosynthase comprising:  
an amino acid sequence of at least about 80% sequence homology to the amino acid sequence set forth in the sequence of SEQ ID NO.1 or  
an amino acid sequence of at least about 80% sequence homology to the amino acid sequence set forth in the sequence of SEQ ID NO.2.
2. The mutant of glycosynthase of claim 1, wherein the one or more mutations are in a peptide region located within residues 176-186, residues 225-237, residues 273-289 in the sequence of SEQ ID NO.1 or  
in a peptide region located within residues 178-188, residues 227-239, residues 275-291 in the sequence of SEQ ID NO.2.
3. The mutant of glycosynthase of claim 2, wherein the residue is substituted with a neutral amino acid, hydrophobic amino acid, acidic amino acid or a basic amino acid,
4. A method for preparing an engineered glycoprotein using the glycosynthase mutant of claims 1-3 comprising coupling of an activated oligosaccharide to a glycoprotein acceptor.
5. The method according to claim 4, wherein the activated oligosaccharide is a glycan oxazoline.
6. The method according to claim 5, wherein the glycan oxazoline comprises an N-glycan having the following formula:



wherein  $R^1$  is -H or N-acetyl glucosamine attached via a  $\beta$ -1,4 linkage, and  $R^2$  and  $R^3$  are same or different and are independently selected from the group consisting of:



7. The method according to claim 4, wherein the glycoprotein acceptor contains a GlcNAc monosaccharide.
8. The method according to claim 4, wherein the glycoprotein acceptor is a non-fucosylated GlcNAc-acceptor.
9. The method according to claim 4, wherein the glycoprotein acceptor is a glycopeptide, a glycoprotein, an antibody or a fragment thereof.
10. The method according to claim 4, wherein the glycoprotein acceptor is a core fucosylated or non-fucosylated GlcNAc-IgG acceptor or a fragment thereof.
11. The method according to claim 10, wherein the GlcNAc-IgG acceptor is derived from a monoclonal antibody targeting Globo series antigen, Her-2, CD20, TNF- $\alpha$ , PD-1, PD-L1, and/or EGFR receptor.
12. The method according to claim 11, wherein the Globo series antigen is Globo H, SSEA-4 and/or SSEA-3.
13. The method of claim 11, wherein the monoclonal antibody is Herceptin (trastuzumab), Perjeta (pertuzumab), Erbitux (cetuximab), Rituxan (rituximab), Vectibix (panitumumab), Humira (adalimumab), Keytruda (pembrolizumab) or Bavencio (avelumab).
14. The method of claim 11, wherein the monoclonal antibody is OBI-888 or OBI-898.
15. A glycoprotein prepared by the method of any one of claims 4-14.

16. A pharmaceutical composition comprising the glycoprotein of claim 15 and a pharmaceutically acceptable carrier or excipient.
17. A method for treating cancer, comprising administering to a subject in need thereof an effective amount of the pharmaceutical composition according claim 16.
18. The method of claim 17, wherein the subject is human.
19. The method of claim 17, wherein the cancer is Globo series antigen expressing cancer.
20. The method of claim 19, wherein the Globo series antigen expressing cancer is selected from the group consisting of sarcoma, skin cancer, leukemia, lymphoma, brain cancer, glioblastoma, lung cancer, breast cancer, oral cancer, head-and-neck cancer, nasopharyngeal cancer, esophagus cancer, stomach cancer, liver cancer, bile duct cancer, gallbladder cancer, bladder cancer, pancreatic cancer, intestinal cancer, colorectal cancer, kidney cancer, cervix cancer, endometrial cancer, ovarian cancer, testicular cancer, buccal cancer, oropharyngeal cancer, laryngeal cancer and prostate cancer.
21. A glycan engineering enzyme comprising one or more functional domains, comprising: an amino acid sequence of SEQ ID NO.1 or SEQ ID NO.2.
22. The enzyme of claim 21, wherein the amino acid sequence is selected from *Streptococcus equi* or *Streptococcus dysgalactiae*.
23. A method for monitoring transglycosylation process, comprising:
  - (a) applying a sample to a UPLC glycoprotein amide column;
  - (b) eluting said high performance liquid chromatography (HPLC) column with a mobile phase to obtain an eluate;
  - (c) obtaining a chromatogram of said eluate;
  - (d) measuring and comparing the absorbance in the test sample; and
  - (e) determining the stage and/or status of transglycosylation;wherein said mobile phase comprises a trifluoroacetic acid-1,1,1,3,3,3-Hexafluoro-2-propanol-water solution and a trifluoroacetic acid-1,1,1,3,3,3-Hexafluoro-2-propanol-acetonitrile solution.
24. The method of claim 23, wherein said eluting is conducted at a flow rate of 0.1 to 10 mL/min.
25. The method of claim 23, wherein the sample is a glycoprotein prepared by the method of any one of claims 4-14.
26. A method for identifying a hemi-glycosylated monoclonal antibody (mAb-1N-G2S2) or a fully-glycosylated monoclonal antibody (mAb-2N-G2S2), comprising:
  - (a) applying a sample to a UPLC glycoprotein amide column;

- (b) eluting said high performance liquid chromatography (HPLC) column with a mobile phase to obtain an eluate;
- (c) obtaining a chromatogram of said eluate;
- (d) measuring and comparing the absorbance in the test sample; and
- (e) determining the stage and/or status of glycosylation;

wherein said mobile phase comprises a trifluoroacetic acid-1,1,1,3,3,3-Hexafluoro-2-propanol-water solution and a trifluoroacetic acid-1,1,1,3,3,3-Hexafluoro-2-propanol-acetonitrile solution.

- 27. The method of claim 26, wherein said eluting is conducted at a flow rate of 0.1 to 10 mL/min.
- 28. The method of claim 26, wherein the sample is Herceptin (trastuzumab), Perjeta (pertuzumab), Erbitux (cetuximab), Rituxan (rituximab), Vectibix (panitumumab), Humira (adalimumab), Keytruda (pembrolizumab) or Bavencio (avelumab).
- 29. The method according to claim 26, wherein the sample is OBI-888 or OBI-898.
- 30. The method of claim 26, wherein the sample is a glycoprotein prepared by the method of any one of claims 4-14.
- 31. A homogeneous glycol-antibody made by the process of claim 4.
- 32. A pharmaceutical composition comprising the population of homogeneous glycol-antibody of claim 31.
- 33. A vector expressing the peptide encoded by amino acid as set forth in SEQ ID. No 1 or No 2.

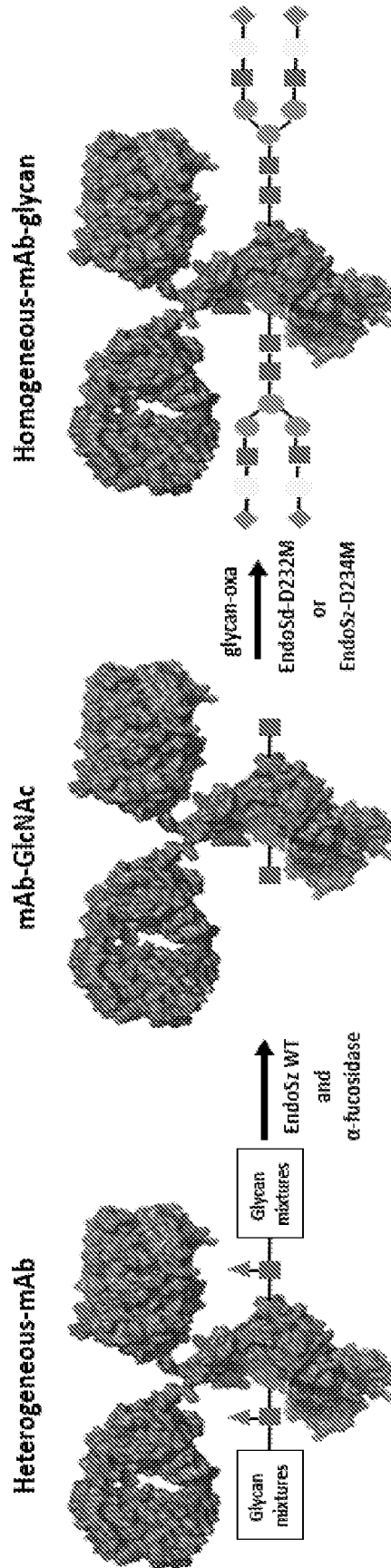


Fig. 1A

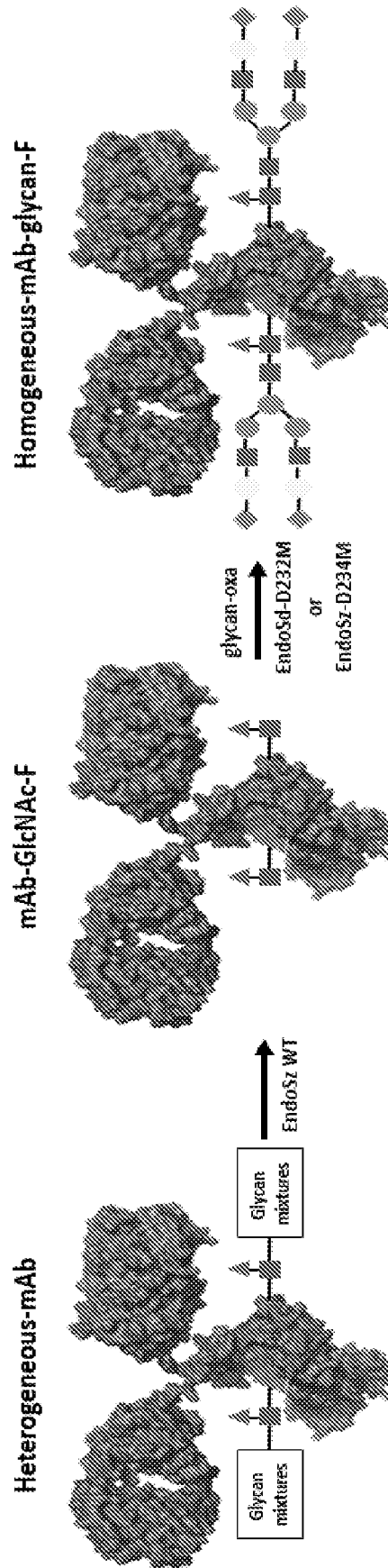


Fig. 1B

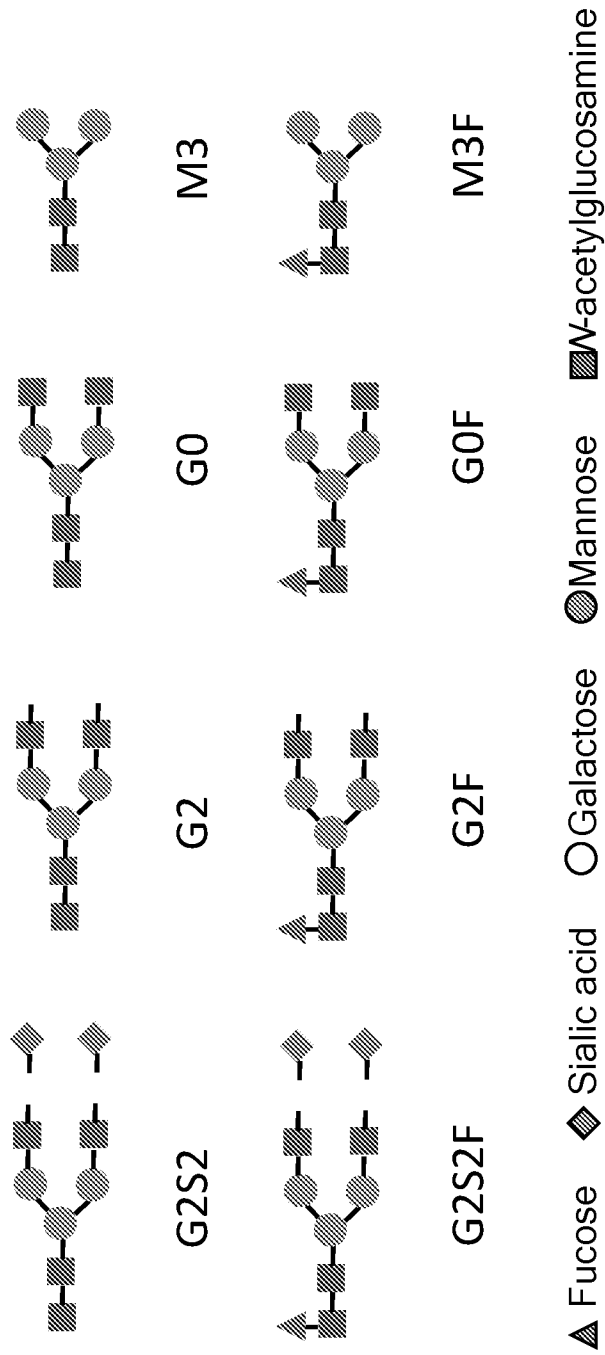
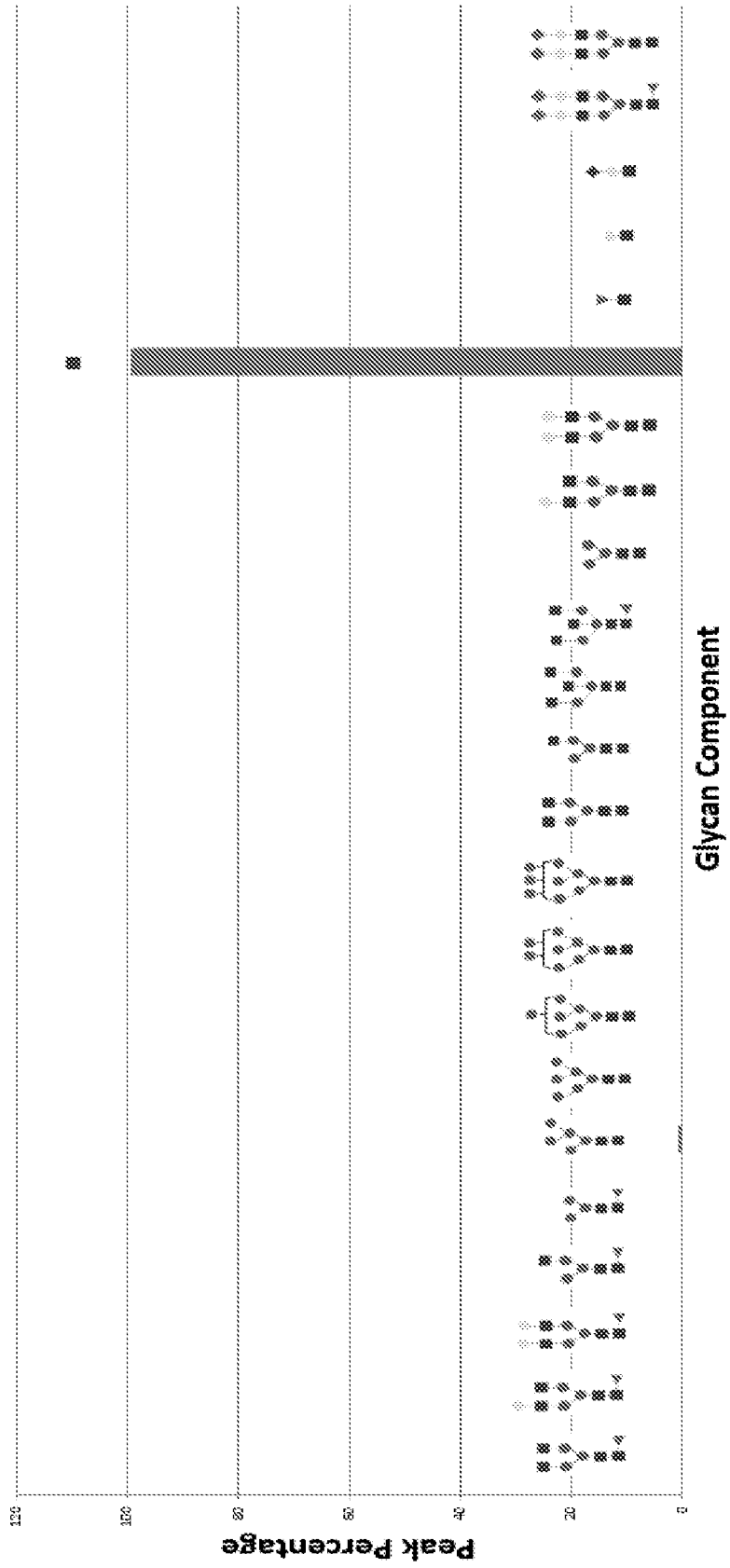


Fig. 1C



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Fig. 2

Figure 3

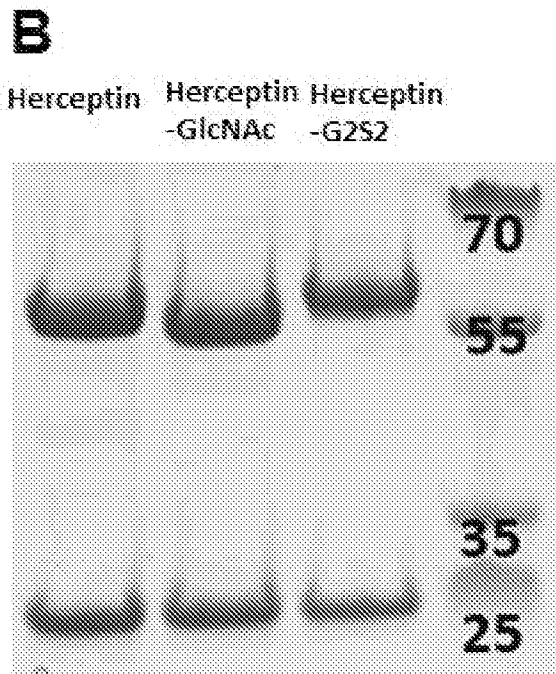
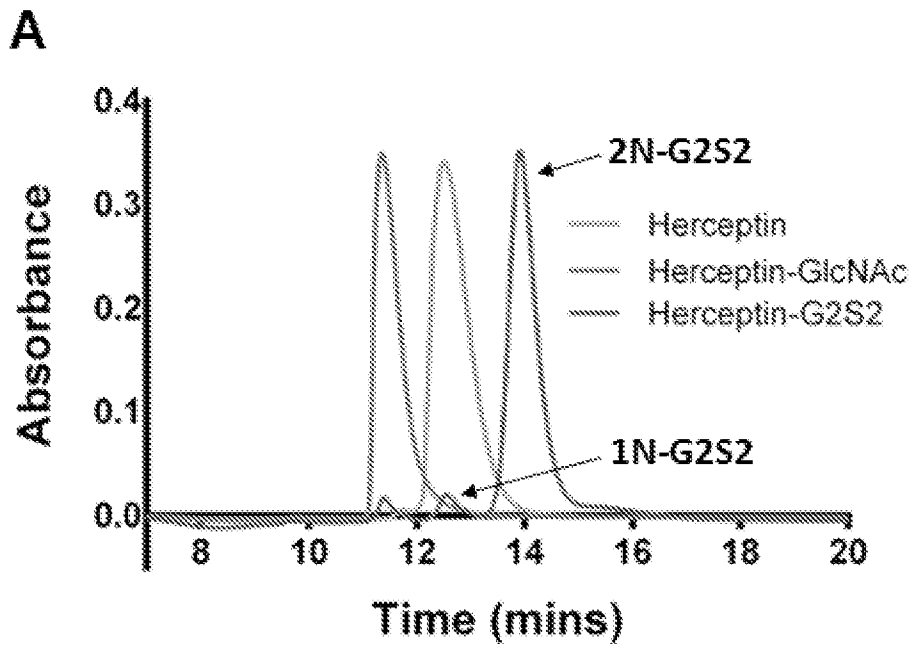




Figure 5

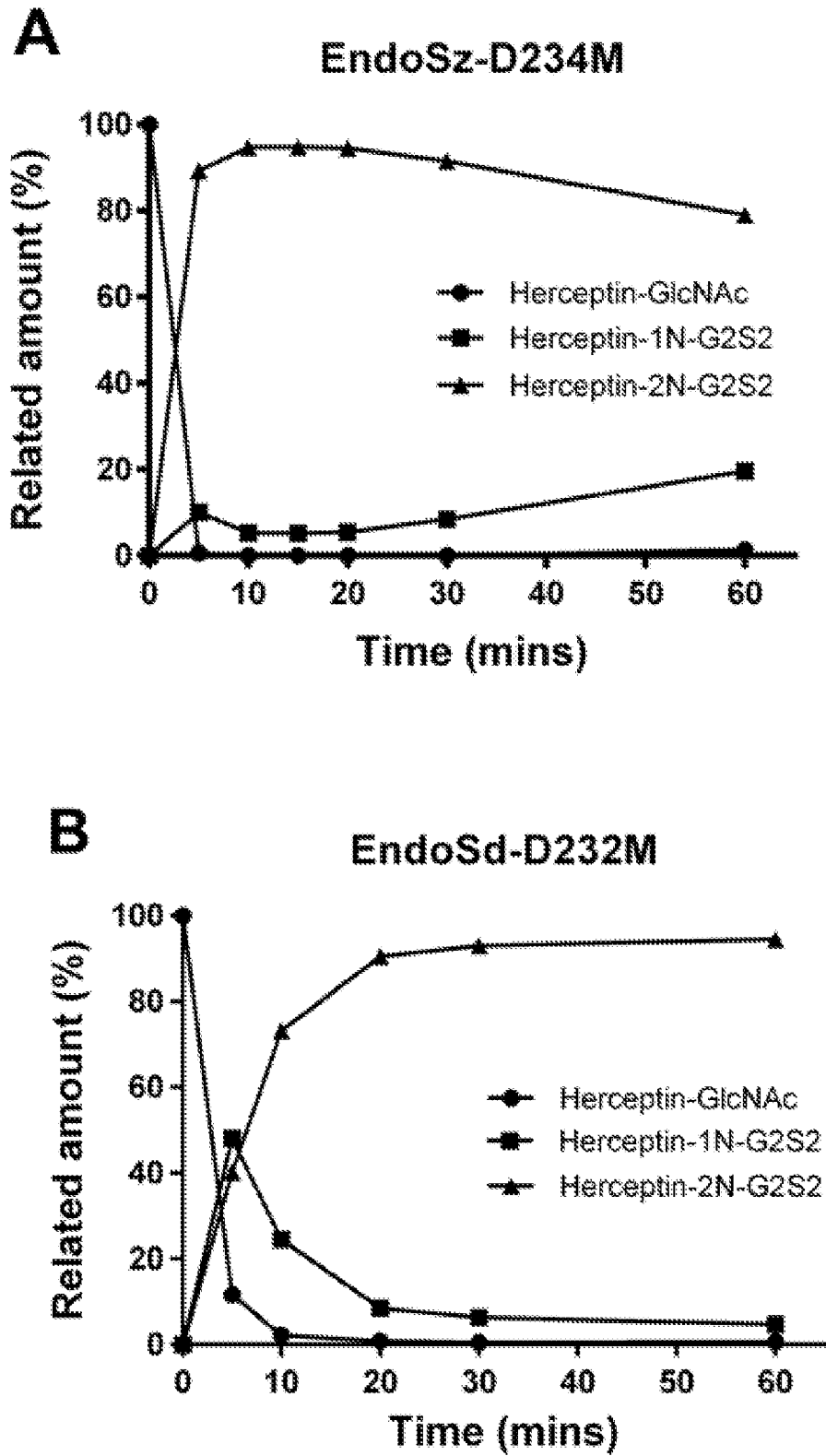


Figure 6

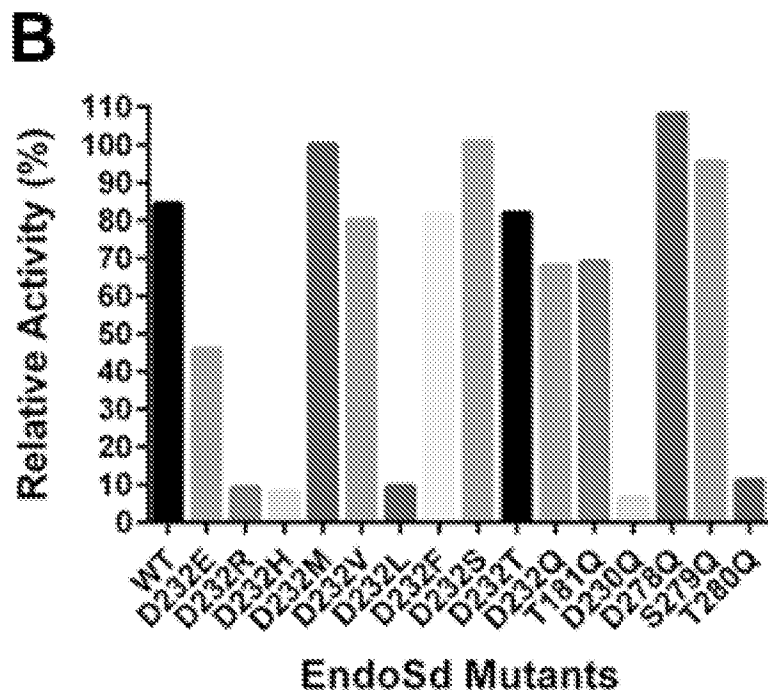
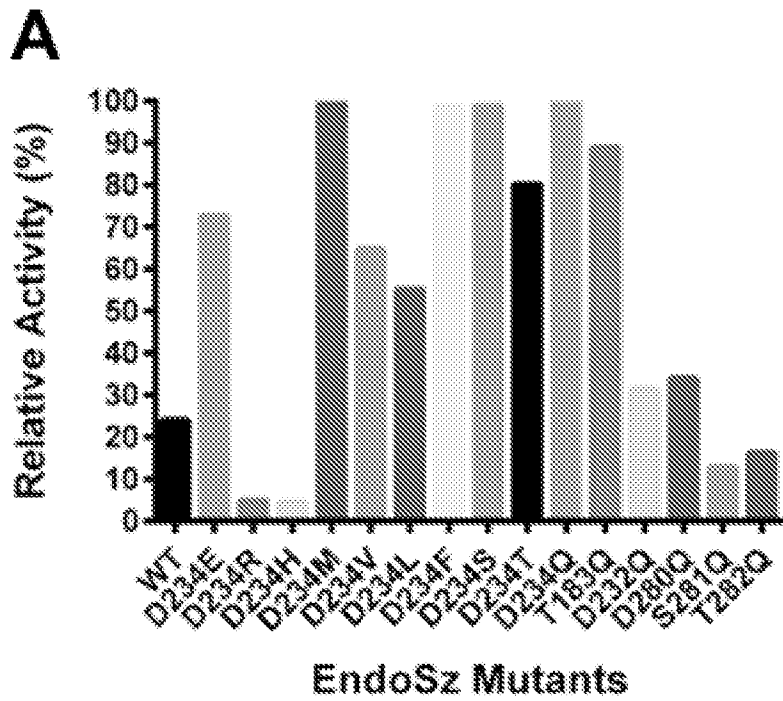
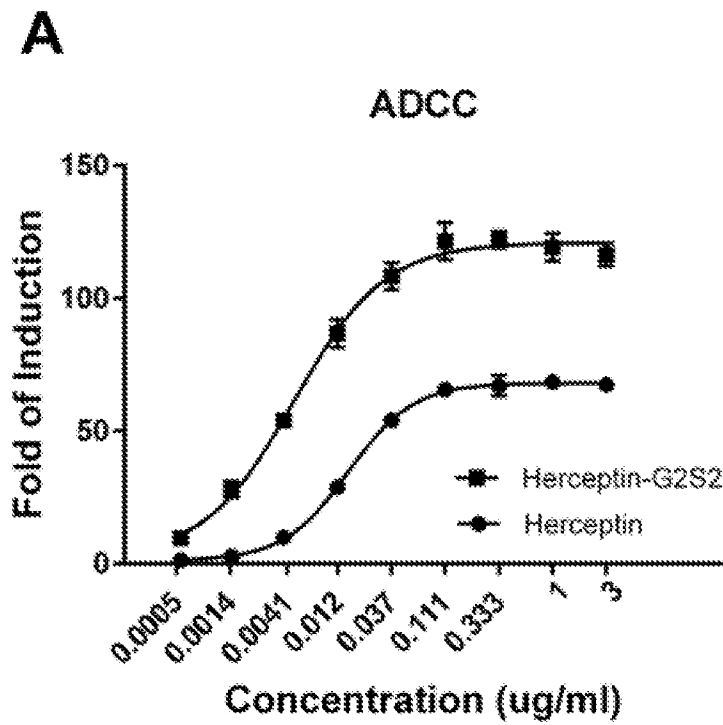


Figure 7



**B**

| Antibody  | GlcNAc | 1N-G2S2 | 2N-G2S2 | ADCC Original (EC 50) | ADCC homogeneou s (EC 50) | ADCC increase Fold |
|-----------|--------|---------|---------|-----------------------|---------------------------|--------------------|
| OBI-888   | 6.33%  | 6.10%   | 87.57%  | 11.92 (ug/ml)         | 0.45 (ug/ml)              | 26.49              |
| Herceptin | 0.44%  | 8.45%   | 91.11%  | 15.29 (ug/ml)         | 5.10(ug/ml)               | 3.00               |
| Perjeta   | 0.00%  | 7.51%   | 92.49%  | 23.19 (ug/ml)         | 3.75 (ug/ml)              | 6.18               |
| Erbbitux  | 2.71%  | 9.38%   | 87.92%  | 8.84 (ng/ml)          | 0.86 (ng/ml)              | 10.28              |
| Rituxan   | 0.00%  | 2.43%   | 97.57%  | 42.4 (ng/ml)          | 3.6 (ng/ml)               | 11.78              |
| OBI-898   | 0.87%  | 9.40%   | 89.73%  | -                     | -                         | -                  |
| Vectibix  | 1.56%  | 13.21%  | 86.12%  | -                     | -                         | -                  |
| Humira    | 0.92%  | 5.40%   | 93.68%  | -                     | -                         | -                  |
| Keytruda  | 7.65%  | 16.54%  | 75.81%  | -                     | -                         | -                  |
| Bavencio  | 2.35%  | 6.95%   | 90.73%  | -                     | -                         | -                  |

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US19/39414

## Box No. 1 Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
- a.  forming part of the international application as filed:  
 in the form of an Annex C/ST.25 text file.  
 on paper or in the form of an image file.
- b.  furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c.  furnished subsequent to the international filing date for the purposes of international search only:  
 in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).  
 on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
2.  In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US19/39414

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3.  Claims Nos.: 4-20, 25, 30-32  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

\*\*\*-Please See Supplemental Page-\*\*\*

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-3, 21, 22, 33; SEQ ID NO: 1

- Remark on Protest**
- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US19/39414

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC - C07K 16/00, 16/18, 16/28; C12N 9/24 (2019.01)  
 CPC - A61P 31/18, 35/00; C07K 14/473, 14/4725, 16/00, 16/18, 16/2809, 16/2887; C12N 1/20, 9/2402; C12P 21/005; C12R 1/46; C12Y 302/01096  
 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)  
 See Search History document  
 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
 See Search History document  
 Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
 See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category*       | Citation of document, with indication, where appropriate, of the relevant passages                                   | Relevant to claim No.     |
|-----------------|--|---------------------------|
| X<br>-----<br>A | US 2015/0087814 A1 (UNIVERSITY OF MARYLAND, BALTIMORE) 26 March 2015; paragraphs [0016], [0057]                      | 1-3<br>-----<br>21-22, 33 |
| A               | US 9,850,473 B2 (UNIVERSITY OF MARYLAND, BALTIMORE) 26 December 2017; column 14, lines 31-40; column 18, lines 44-57 | 21-22, 33                 |
| A               | WO 2017/010559 A1 (DAIICHI SANKYO COMPANY, LIMITED) 19 January 2017; page 5, fourth paragraph                        | 21-22, 33                 |

Further documents are listed in the continuation of Box C.  See patent family annex.

\* Special categories of cited documents:  
 "A" document defining the general state of the art which is not considered to be of particular relevance  
 "D" document cited by the applicant in the international application  
 "E" earlier application or patent but published on or after the international filing date  
 "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)  
 "O" document referring to an oral disclosure, use, exhibition or other means  
 "P" document published prior to the international filing date but later than the priority date claimed  
 "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  
 "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone  
 "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art  
 "&" document member of the same patent family

Date of the actual completion of the international search  
18 November 2019 (18.11.2019)  
 Date of mailing of the international search report  
**03 DEC 2019**

Name and mailing address of the ISA/US  
 Mail Stop PCT, Attn: ISA/US, Commissioner for Patents  
 P.O. Box 1450, Alexandria, Virginia 22313-1450  
 Facsimile No. 571-273-8300  
 Authorized officer  
 Shane Thomas  
 Telephone No. PCT Helpdesk: 571-272-4300

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

International application No.

PCT/US19/39414

\*\*\*-Continued from Box No. III Observations where unity of invention is lacking: \*\*\*-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+, Claims 1-3, 21, 22, 33 and SEQ ID NO: 1 (mutant glycosynthase) are directed toward a mutant glycosynthase glycan engineering enzyme and vector thereof.

The mutant glycosynthase and vector will be searched to the extent they encompass SEQ ID NO: 1 (first exemplary mutant glycosynthase sequence). Applicant is invited to elect additional glycosynthase sequence(s), with specified SEQ ID NO: for each, or with specified substitution(s) at specified site(s) of a SEQ ID NO:, such that the sequence of each elected species is fully specified (i.e. no optional or variable residues or substituents), where available as an option within at least one searchable claim, to be searched. Additional glycosynthase sequence(s) will be searched upon the payment of additional fees. It is believed that claims 1-3 (each in-part), 2 (in-part), 22 (in-part), and 33 (in-part) encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NO: 1 (mutant glycosynthase sequence). Applicants must specify the searchable claims that encompass any additionally elected glycosynthase sequence(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be SEQ ID NO: 2 (mutant glycosynthase sequence).

Group II, Claims 23-24 and 26-29 are directed toward methods for monitoring a transglycosylation process, and identifying a hemi-glycosylated or fully glycosylated antibody.

The inventions listed as Groups I+ and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Groups I+ include SEQ ID NO: 1, not present in Group II; the special technical features of Group II include high performance liquid chromatography, not present in any of Groups I+.

Groups I+ and II share the technical features including: glycosylation, transglycosylation, or glycan engineering.

However, these shared technical features are previously disclosed by US 9,850,473 B2 to University of Maryland, Baltimore (hereinafter 'Maryland').

Maryland discloses glycosylation (transglycosylation; abstract).

No technical features are shared between the mutant glycosynthase sequences of Groups I+ and, accordingly, these groups lack unity a priori.

Groups I+ share the technical features including: a mutant of glycosynthase comprising: an amino acid sequence; a glycan engineering enzyme comprising one or more functional domains; and a vector expressing the polypeptide.

However, these shared technical features are previously disclosed by Maryland, as above.

Maryland discloses a mutant of glycosynthase (mutant enzymes with increased transglycosylation activity (a mutant of glycosynthase); abstract) comprising: an amino acid sequence (comprising: an amino acid sequence; column 4, lines 41-45); a glycan engineering enzyme comprising one or more functional domains (a glycan engineering enzyme comprising one or more catalytic (functional) domains; column 18, lines 44-57); and a vector expressing the polypeptide (a vector expressing the polypeptide; column 14, lines 31-40).

Since none of the special technical features of the Groups I+ and II inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the Maryland reference, unity of invention is lacking.