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REVIEW

Humanizing glycosylation pathways in eukaryotic expression systems

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Abstract Glycosylation represents the most widespread posttranslational modifications, found in a broad spectrum of natural and therapeutic recombinant proteins. It highly affects bioactivity, site-specificity, stability, solubility, immunogenicity, and serum half-life of glycoproteins. Numerous expression hosts including yeasts, insect cells, transgenic plants, and mammalian cells have been explored for synthesizing therapeutic glycoproteins. However, glycosylation profile of eukaryotic expression systems differs from human. Glycosylation strategies have been proposed for humanizing the glycosylation pathways in expression hosts which is the main theme of this review. Besides, we also highlighted the glycosylation potential of protozoan parasites by emphasizing on the mammalian-like glycosylation potential of *Leishmania tarentolae* known as *Leishmania* expression system.

Keywords Glycosylation pattern · Glycoengineering · LEXSY · Eukaryotic expression systems

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Introduction

Among posttranslational modifications (PTMs), glycosylation is the most common and complex modification of many cell surface and secreted eukaryotic proteins. Glycosylation is the enzymatic addition of oligosaccharides to nascent polypeptide chains in the endoplasmic reticulum (ER). Attached oligosaccharide structure is further modified by an array of glycosidases and glycosyltransferases inside ER and Golgi complex. The modification reactions occurring in ER are highly conserved between lower and higher eukaryotes. While reactions taking place inside Golgi complex varies among species and cell types (Jacobs and Callewaert 2009). Due to non-template based biosynthesis of glycans, glycoproteins typically occur as a mixture of glycoforms. Consequently, making the field of glycoproteomics more complex compared to other omics (Zoldoš et al. 2013). Approximately, 50% of the human native proteins including immunoglobulin are glycoproteins. The glycan residues greatly influence the physical and chemical properties of proteins i.e. folding, site specificity, cellular homeostasis, and immune regulation (Dalziel et al. 2014). Nonhuman glycans make recombinant proteins immunogenic (Li and d'Anjou 2009). That's why several academic and industrial laboratories have focused on engineering the glycosylation pathways of expression systems for humanizing the glycosylation reactions and eliminating immunogenic epitopes.

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Recent insights in glycan analysis and genotyping technologies have unveiled glycogenes, encoding glycosyltransferases, glycosidases, and other proteins that were involved in glycan biosynthesis (Kawamura et al. 2008; Lauc and Zoldoš 2010). In addition, several high-throughput glycomics projects have been launched to decipher the role of carbohydrates in living system (Aoki-Kinoshita 2013; Hashimoto et al. 2006; Hirabayashi 2004; Struwe et al. 2016). For example, by using glycomeDB database (http://www.glycome-db.org/), which stores structural information of carbohydrates, one can get an overview and compare carbohydrate structures in different databases.

The glycosylation profile of recombinant proteins, destined for human use, is of critical significance. Because glycosylation controls the biological activity, function, clearance from blood stream, and antigenicity of recombinant proteins. The glycosylation profile of nonhuman cells is extremely different from human especially, those which are more distant to human in evolutionary terms i.e. bacteria, yeasts, insects, and plants (Fig. 1) (Anyaogu and Mortensen 2015; Gomord et al. 2005; Harrison and Jarvis 2006; Li and d'Anjou 2009). Although *E. coli* is the most prominent expression host in biotechnology industry. However, lack of natural PTMs machinery hinders its use for glycoproteins. Detection antibodies for diagnostic purposes (Khan and Sadroddiny 2016; Rahmatpour et al. 2016), aglycosylated proteins, or those whose quality, safety, efficacy, and half-life are not affected in the absence of glycosylation can be produced in *E. coli*. Well understood genome and low production cost have motivated researchers to equip *E. coli* with human-like glycosylation reactions (Naegeli et al. 2014). However, more efforts are needed to enable *E. coli* for the production of therapeutic glycoproteins. In contrast, eukaryotic expression systems including yeasts, insect cells, transgenic plants, mammalian cells, and parasites possess glycosylation machinery. Amid, the molecular structure and biochemical properties of recombinant proteins produced by mammalian cell lines are almost similar to human. This characteristic feature makes mammalian cells ideal expression platform for the production of commercially available recombinant glycoproteins (Swiech et al. 2012; Zhu 2012).

Numerous strategies have been proposed for tailoring the glycosylation pathways and humanizing the glycosylation profile of expression hosts (Rich and Withers 2009). In this review, we discussed glycosylation strategies that have been reported for producing human-like glycoproteins in yeasts, insect cells, transgenic plants, and mammalian cells. Moreover, we also highlighted the mammalian-like glycosylation potential of *Leishmania* expression system (LEXSY).

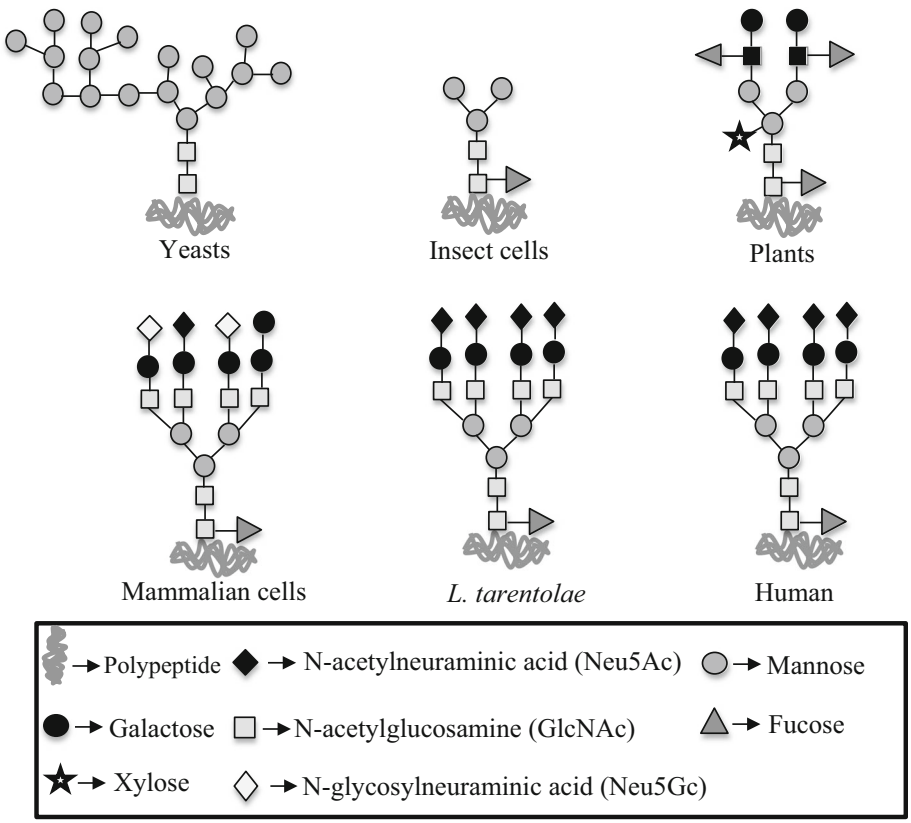

Fig. 1 Comparison of glycosylation profile of recombinant glycoproteins derived from yeasts, insect cells, and *L. tarentolae*, mammalian cells, and plants versus human beings

Tailoring eukaryotic expression systems

Glycoengineering in yeasts

Ease of handling, better-understood genome, rapid growth, availability of numerous expression vectors together with PTMs machinery make yeasts promising expression candidates. Plethora of yeast species including *Saccharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*, *Kluyveromyces lactis*, *Schizosaccharomyces pombe*, *Yarrowia lipolytica*, and *Arxula adeninivorans* have been explored for heterologous expression (Baghban et al. 2016; Damasceno et al. 2012; Theron et al. 2014). Of which, *P. pastoris* and *S. cerevisiae* have become the most suitable and powerful expression platform. However, unfortunately, yeasts-derived recombinant proteins contain *N*-glycans with high-mannose residues, which may elicit adverse immune reactions upon long term administration in human. In addition, hypermannosylation also reduces serum half-life and compromises the efficacy of most therapeutic glycoproteins (Wildt and Gerngross 2005). Since long, efforts have been made to humanize the glycosylation pathways in yeasts and improve folding, trafficking, and secretion of recombinant proteins (Hou et al. 2012).

Glycosylation reactions inside the ER of yeasts and mammals are same. Variations originate inside Golgi bodies. In the yeasts Golgi, the activity of Outer Chain elongation (*OCH1*) encoded α1,6-mannosyltransferase (Och1p) triggers hypermannosylation of secreted proteins at a great heterogeneity. It extends the outer chain of *N*-linked oligosaccharides up to 100 mannoses or more (Dean 1999). The first reaction in hypermannosylation is catalyzed by Och1p. Therefore, attempts have been made to delete *OCH1* gene from *P. pastoris* and develop a new strain for the production of recombinant proteins with homogeneous shorter glycans (Krainer et al. 2013). The number of mannoses in glycans of knockout strain reduced from ten to eight compared to a wild type strain. However, like previously developed knockout *S. cerevisiae* (Nagasu et al. 1992), *P. pastoris* also demonstrated poor budding, thermo-sensitivity, increased flocculation, and slow growth (Krainer et al. 2013). In one other study, a triple mutant *S. cerevisiae* strain has been generated by disrupting *ALG3*, *OCH1*, and *MNN1* genes (He et al. 2014). The growth-defect phenotype was overcome by adoptive evolution. Resultant triple mutant strain produced Man5GlcNAc2 intermediate of human *N*-glycosylation without revealing any growth defects. This mutant strain could be used as an initial strain to generate a yeast-based therapeutic glycoprotein expression system. Genome editing tool, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) systems provide an extremely efficient system for targeted gene disruption. This technology has facilitated the targeted inactivation of genes in vitro and in vivo in a broad range of organisms (Ablain et al. 2015). CRIPR/Cas9 has been employed in *S. cerevisiae* for genome engineering (DiCarlo et al. 2013). The approach was simple and powerful, and allowed site-specific mutagenesis and allelic replacement in yeasts efficiently.

Moreover, several combinatorial genetic libraries, composed of an array of different fusion protein constructs, have also been reported for humanizing glycosylation in *P. pastoris* (Nett et al. 2011). In this method, each construct was accompanied by a fungal cellular targeting sequence, fused in-frame to a catalytic domain of heterologous glycosylation enzyme. The glycosylation profile of which was analyzed by a 96-well high-throughput protein expression protocol. Although combinatorial genetic approach along with high throughput screening protocol allowed the production of glycoproteins with complex *N*-glycans in *P. pastoris*. Nevertheless, this approach was unable to predict precisely the practical optimal activity of leader or catalytic domain in vivo.

The focus of previous glycoengineering strategies was to generate a substrate for Golgi-localized glycosyltransferases by tailoring lipid-linked oligosaccharide (LLO) biosynthesis pathway. However, recent studies have shown that LLO modification often resulted in the formation of intermediate structures, leading to hypoglycosylation of target proteins. However, by expressing protozoans oligosaccharyltransferases in yeasts, one can overwhelm hypoglycosylation and produce safe biologically active glycoproteins (Piirainen et al. 2014). Along with human-like glycosylation, the number of *N*-glycans at specific sites is also essential for the biological activity of recombinant proteins. For instance, native human erythropoietin (EPO) contains three glycosylation sites. An aglycosylated form of EPO produced in *E. coli* confers short half-life in vivo while a hyperglycosylated darbepoietin alfa, carrying two additional *N*-glycans exhibits a threefold increase in serum half-life than EPO (Sinclair and Elliott 2005). Compared to LLO modification, expressing protozoans' oligosaccharides in yeasts could be more beneficial for improving site occupancy and ensuring appropriate number of *N*-glycans in recombinant proteins. Because protozoans oligosaccharyltransferases possess alter specificities for both oligosaccharide and protein acceptor site.

Glycoengineering in insect cells

Baculoviruses belonging to *Baculoviridae* family are lytic viruses of insects, but innocuous to human. Insect derived-baculovirus vector system (baculovirus expression vector

system, BEVS) (Smith et al. 1983) has been used for producing several hundred recombinant proteins, ranging from cytosolic enzymes to membrane-bound proteins (van Oers et al. 2015). Characteristic features: biosafety, ease of manipulation, ability to carry large DNA sequences, low cytotoxicity, non-replicative nature in transduced cells, and high protein titers make BEVS an ideal expression approach (Chen et al. 2011; Kost et al. 2005). By employing BEVS, insect cells can produce recombinant proteins with a variety of *O*- and *N*-glycan structures excluding sialylation (Marchal et al. 2001), which is essential for serum half-life and biological activity of many glycoproteins e.g. EPO and some antibodies (Geisler and Jarvis 2012). In fact, insect cells produce a small amount of most common sialic acid nucleotide, cytidine monophosphate (CMP)-sialic acid (CMP-Neu5Ac), an essential substrate for sialyltransferases. This enzyme catalyzes the transferring of sialic acid from donor substrate to acceptor oligosaccharides. Studies have made it possible to increase the intracellular pool of CMP-Neu5Ac by cloning human CMP-sialic acid synthase in insect cells using BEVS (Lawrence et al. 2001). Yet, the approach was expensive. Because for efficient sialylation the culturing medium of engineered BEVS/insect cell line had to be supplemented with an expensive sialic acid precursor, *N*-acetylmannosamine (ManNAc). By generating endogenous sialic acid precursor pool, one can overwhelm the use of expensive precursor in culturing medium. For this purpose, *N*-acetylglucosamine-6-phosphate 2′-epimerase (GNPE) enzyme of *E. coli* has been proposed in insect cells. In sialic acid degradation pathway, GNPE enzyme normally converts *N*-acetyl-D-mannosamine-6-phosphate (ManNAc-6-P) to *N*-acetyl-D-glucosamine-6-phosphate (GlcNAc-6-P). In insect cells, GlcNAc-6-P is more, because it is produced from sugar in normal metabolic process. Whereas, ManNAc-6-P is present in negligible amount. It was assumed that GNPE could work in reverse manner i.e. producing ManNAc-6-P from GlcNAc-6-P. To validate the assumption, GNPE was expressed together with recombinant protein in *Spodoptera frugiperda* (Geisler and Jarvis 2012). This architecture allowed engineered cells to produce endogenous CMP-sialic acid pool efficiently and resulted in recombinant glycoprotein with terminal sialylation even in the absence of exogenous precursor.

Together with mammalian-like α1,6-fucose, insect derived recombinant proteins also carry α1,3-fucose, a glycan epitope that may elicit IgE-mediated adverse events in hypersensitive population (Harrison and Jarvis 2006; Palmberger et al. 2014). Literally, fucosylation is driven by a key donor substrate GDP-L-fucose. By demolishing GDP-L-fucose, one can eliminate undesired epitope. Studies have demonstrated that *Pseudomonas aeruginosa* encodes guanosine-5′-diphospho (GDP)-4-dehydro-6-deoxy-D-mannose reductase (RMD) enzyme, which converts precursor of GDP-L-fucose into a dead end product. Thus, RMD has been cloned in BEVS for producing rituximab, a nonfucosylated therapeutic antibody. Like Chinese hamster ovary (CHO) cells, deficient in fucose de novo pathway (von Horsten et al. 2010), nonfucosylated rituximab revealed enhanced effector activity in vivo (Mabashi-Asazuma et al. 2014).

Both insect and mammalian cells produce a common intermediate structure, Manα6(GlcNAcβ2Manα3)-Manβ4GlcNAcβ4GlcNAc-R. Mammalian cells elongate this intermediate product to complex *N*-glycans by various glycosyltransferases. In contrast, insect cells fail to elongate this product, and convert it into paucimannose *N*-glycans by a membrane bound *β-N*-acetylglucosaminidase enzyme (Geisler et al. 2008). Attempts have been made to block the activity of this enzyme by silencing *fused lobes* (*fdl*) gene through RNA interference (RNAi) (Geisler et al. 2008; Kim et al. 2012). The synergistic effects of expressing β1,4-galactosyltransferase (βGalT) and suppressing *fdl* have also been investigated in *Drosophila* S2 cells (Kim et al. 2011). Despite partial suppression of *fdl*, glycoengineered S2 cells secreted fully glycosylated EPO. Only sialylation of *N*-glycans was missing. Complete suppression can also be achieved by using gene knockout strategies and/or in vivo transcription of antisense RNA (Kim et al. 2011). In addition, CRISPR–Cas9 system has also been reported for editing *fdl* gene in S2 cells. This system efficiently generated site-specific nucleotide insertions and deletions and reduced insect-type paucimannose products. Resulting S2 cells produced partially elongated, mammalian-type complex *N*-glycans, ranging from Man5 to Man9 (Mabashi-Asazuma et al. 2015). Further technological advances will have a significant impact on manufacturing processes, which may offer new class of glycoprotein therapeutics with customized functions.

Glycoengineering in plants

Plants have been utilized as a suitable alternative to microbial and animal cell factories for producing clinically useful recombinant proteins. Together with the production of biologically active mammalian proteins in high titers at low cost, plants have the potential to perform intricate PTMs (Faye et al. 2005; Gomord and Faye 2004). Like other eukaryotes, *N*-glycosylation pathways in plants emerge in ER, where oligosaccharide precursor Glc3Man9GlcNAc2 is converted to Man8GlcNAc2. Up to the formation of vital intermediate GlcNAc2Man3GlcNAc2, all *N*-glycan processing steps in plants are virtually identical to mammals. Complex-type *N*-glycans in plants and mammals are produced after trimming the precursors, followed by the addition of several sugar residues by

glycosyltransferases. However, glycosyltransferases are organism specific. This result in structurally different biantennary complex-type *N*-glycans in plants and mammals (Castilho and Steinkellner 2012; Saint-Jore-Dupas et al. 2007).

In plants, the proximal *N*-acetylglucosamine of core is replaced by an α1,3-fucose and β-mannose by a bisecting β1,2-xylose. While in mammals *N*-acetylglucosamine and β-mannose of core are substituted by an α1,6-fucose and β1,4-*N*-acetylglucosamine respectively (Saint-Jore-Dupas et al. 2007). The attachment of these undesired structures, which make recombinant proteins potentially immunogenic, are catalyzed by α1,3-fucosyltransferase and β1,2-xylosyltransferase (Jin et al. 2006, 2008). The synthesis of undesired epitopes can be eliminated by employing gene knockout strategies: site directed mutagenesis, homologous recombination, and RNAi. A gene knockout *Physcomitrella patens*, generated by homologous recombination was able to produce epitopes-free *N*-glycans (Koprivova et al. 2004). Under standard conditions, the new strain did not exhibit any change in phenotype and morphology compared to the wild-type strain. In glycoengineered rice, same epitopes have been down-regulated by using RNAi technology (Shin et al. 2011). When the *N*-glycans from RNAi cell lines were compared with those isolated from wild-type cell suspension, a significant decrease in core α1,3-fucosylated and/or β1,2-xylosylated glycans was observed. Similar to previous gene knockout cell lines (Koprivova et al. 2004), resulting rice cell lines did not demonstrate any change in cell division, proliferation, and protein secretion aptitude. In another study, epitopes-free monoclonal antibody (mAb) has been produced in *Lemna minor* by coexpressing the gene with RNAi construct. Resultant cell lines produced a mAb devoid of plant-specific *N*-glycans. Like defucosylated mAbs expressed in CHO cells (Shinkawa et al. 2003), the mAb was biological active and demonstrated 20- to 30-fold enhancement in antibody-dependent cell-mediated cytotoxicity (ADCC) (Cox et al. 2006).

Another characteristic feature of glycoproteins is terminal sialylation which is performed by a key enzyme βGalT. Unfortunately, this enzyme is not present in plants. Attempts have been made to express human βGalT in tobacco BY2 cells (Fujiyama et al. 2007). A mAb with galactose-extended glycans was produced by engineered BY2 cells. However, recent insights have illustrated that despite the presence of galactose on *N*-glycans, which serves as an acceptor substrate, terminal sialylation is particularly difficult to accomplish. Because plants lack other essential prerequisites such as, biosynthetic pathway for synthesizing CMP-Neu5Ac, cargo for delivering CMP-Neu5Ac into Golgi, and sialyltransferase for transferring sialic acid from CMP-Neu5Ac to terminal galactose of nascent polypeptide. For in vivo terminal sialylation, several mammalian genes for sialylation have been coexpressed along with the gene for mAb in *Nicotiana benthamiana*. A mAb with sialic acid at the Fc domain was produced, which indicated full integrity and neutralization potential for the target protein (Castilho et al. 2010; Paccalet et al. 2015). Besides, bisected and branched *N*-glycans are also needed for glycoproteins. *N. benthamiana*, deficient in plant-specific glycosylation but containing β1,4-mannosyl-β1,4-*N*-acetylglucosaminyltransferase (GnTIII), α1,3-mannosyl-β1,4-*N*-acetylglucosaminyltransferase (GnTIV), and α1,6-mannosyl-β1,6-*N*-acetylglucosaminyltransferase (GnTV) has been explored for heterologous expression. Coexpression of GnTIII, GnTIV, and GnTV resulted in the efficient production of bisected, tri-, and tetraantennary complex *N*-glycans (Castilho et al. 2011). So far, outstanding success has been achieved in tailoring plant glycosylation reactions for the production of humanized glycoproteins. Recent advances in glycoengineering strategies will enable plants to produce safe glycoproteins with branched and sialylated *N*-glycans for therapeutic purposes.

Glycoengineering in mammalian cells

Human-like PTMs and protein assembling procedures have made mammalian cell lines the most promising expression platform. More than half of the recombinant proteins available on the market and several hundred candidates in clinical development have been obtained from mammalian cell lines (Zhu 2012). Plethora of mammalian cell lines including, CHO cells, murine myeloma lymphoblastoid-like cells (NS0 and Sp2/0-Ag14), human embryonic kidney 293 (HEK 293) cells, and baby hamster kidney cells (BHK-21) have been explored for heterologous expression (Bazl et al. 2007; Böhm et al. 2015; Chen et al. 2014; Majors et al. 2008; Shabani et al. 2010). Incredible progress in recombinant DNA technology and growing understanding of glycosylation pathways have motivated researchers to manipulate cellular pathways in mammalian cells. Because these cells can produce high protein titers i.e. 50 mg/L to 5–10 g/L (Lim et al. 2010; Rahimpour et al. 2013, 2016).

The glycosylation profile of mammalian cell lines is almost similar to human. However, subtle variations in glycan structures of human and other mammals do persist. Numerous cell line engineering strategies have been proposed for CHO cells to enhance PTMs such as, protein glycosylation and sialylation (Lim et al. 2010). The genome of CHO cell lines represents a valuable tool in glycoengineering but unfortunately, it was unavailable to the biotechnology industry for decades. Today, a publically available annotated genome sequence for CHO cells can be used as a tool in the bioprocessing toolbox (Xu et al. 2011).

The availability of genome sequence may facilitate genome-scale science for the optimization of human-like therapeutic glycoprotein production. Moreover, it will improve product titers, elucidate components of underlying poorly characterized phenotypes, and allow the development of novel omics tools for CHO and other cell lines.

N-glycans of native human glycoproteins contain α2,3- and α2,6-linked terminal sialic acid while recombinant proteins produced by CHO cells contain α2,3-linkage only. In human, α2,6-sialylation of terminal galactose residues is catalyzed by β-galactosyl α2,6-sialyltransferase (ST6Gal). Although homologs exist in CHO cells for human ST6Gal genes. However, transcriptome data show no evidence for their expression (Xu et al. 2011). Regardless of the previous reports, cDNA of ST6Gal I derived from CHO cells has been cloned in antibody producing CHO cell line (Onitsuka et al. 2012). HPLC analysis and sialidase digestion confirmed α2,6-sialylation in about 70% of *N*-linked oligosaccharides. The altered glycan ratios reported in previous studies (Onitsuka et al. 2012) can be overcome by overexpressing ST6Gal 1 in CHO cells by using plasmid expression vector (Lin et al. 2015b). Overexpression of ST6Gal 1 produced recombinant proteins with increased sialylation and human-like glycans. This versatile cell line could be used in biopharmaceutical industry after optimizing growth during clone selection or upstream process development.

In addition, bisected oligosaccharides present on the Fc region of mAbs are also essential for the ADCC. βGnTIII catalyzes the addition of a bisecting oligosaccharides to *N*-glycans. To enhance the ADCC of anti-CD20 antibody, βGnTIII from rat origin has been coexpressed in CHO cell line (Davies et al. 2001; Schuster et al. 2005). The presence of bisecting oligosaccharides was confirmed by HPLC. The antibody was biologically active and killed target cells efficiently even in low concentration. Enhanced ADCC activity of mAbs at lower concentration could be useful in lymphoma and leukemia, expressing small amount of antigen molecules. By exchanging the localization domain of βGnTIII with other Golgi-localized enzymes, one can enhance the expression of bisecting oligosaccharides (Ferrara et al. 2006). The chimeric version of βGnTIII efficiently produced antibody with bisected defucosylated glycans, which can be employed for modulating biological activities of antibodies for therapeutic application.

Unlike other expression hosts, CHO cells also express additional carbohydrate epitopes including the terminal α1,3-galactose (α-Gal). The α-Gal antigen is similar in structure to the epitope of gut bacteria against which anti-α-Gal antibodies are universally present in human blood. Hence, this antigen can react with circulating anti-α-Gal antibodies. Typical biopharmaceutical manufacturing cell lines such as, SP2 and NSO contain biosynthetic machinery for this epitope (Macher and Galili 2008). The majority of immune-related adverse events associated with the licensed mAb Erbitux (cetuximab), synthesized in a murine cell line have been attributed the presence of α-Gal residue (Chung et al. 2008). Even though, CHO cells lack biosynthetic machinery for α-Gal epitope. However, contrary to this, studies have identified an ortholog of *N*-acetyllactosaminide 3-α-galactosyltransferase-1 for α-Gal epitope in CHO cells. The product of which tweaks glycoproteins with α-Gal antigen. For example, a commercial therapeutic protein abatacept (Orencia), expressed in CHO cell lines, contain α-Gal antigen (Bosques et al. 2010). Furthermore, mammalian cell lines synthesize a nonhuman sialic acid, *N*-glycolylneuraminic acid (Neu5Gc), a hydroxylated form of Neu5Ac (Ghaderi et al. 2010). Because of no adverse events in healthy individual, this documented contamination was ignored in the past. However, recent observations have identified anti-Neu5Gc antibodies in human circulation, sometimes at high levels. Cetuximab containing Neu5Gc moiety reacts with anti-Neu5Gc antibodies in the blood thereby, generating immune complexes (Ghaderi et al. 2010). Additionally, unwanted epitopes exert short half-life to therapeutic glycoproteins consequently, reducing the efficacy of therapeutic proteins. Two enzymes, α1,3-galactosyltransferase (Ggta1) and CMP-Neu5Ac hydroxylase (Cmah) have been identified that were involved in synthesizing unwanted contaminants. Cmah catalyzes the conversion of Neu5Ac to its hydroxylated derivative Neu5Gc. While Ggta1 attaches galactose residue to a galactose on *N*-glycans through an α1,3-glycosidic bond subsequently, producing an α-Gal moiety. A CHO cell line deficient in Cmah and Ggta1 sequences allowed the production of epitopes-free recombinant proteins (Lin et al. 2015a).

IgG1 antibodies produced in CHO cells contain high amount of α1,6-fucose at the innermost GlcNAc, which reduces ADCC and inhibits therapeutic antibody function in vivo. The addition of undesired fucose is catalyzed by Fut8, α1,6-fucosyltransferase. FUT8 negative CHO cells have been developed by targeting the catalytic domain of FUT8 gene through Zinc-finger nucleases (ZFNs) (Malphettes et al. 2010). ZFN-derived FUT8 (−/−) cells demonstrated no change in growth profile and produced antibodies devoid of core fucosylation. CRISPR–Cas9 system has also been reported in disrupting the FUT8 gene (Sun et al. 2015). This system demonstrated higher targeting efficiency compared to homologous recombination and ZFNs. Resultant FUT8 (−/−) clone produced defucosylated therapeutic mAb without detrimental changes in cell growth, viability, or product quality. Hence, it could be used in biotechnology industry for manufacturing therapeutic glycoproteins.

Protozoan expression systems

The Trypanosomatidae encompasses a wide range of protozoan parasites including, *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania* species. These parasites are transmitted by insect vectors to human and animals consequently, invading different tissues and cell types. The cell surface of protozoans is covered by glycoconjugates such as, glycosylphosphatidylinositol (GPI)-anchored glycoproteins, GPI-anchored lipophosphoglycan and a class of free GPI glycolipids (Ilgoutz et al. 1999). GPI-anchored glycoproteins protect parasites from alternative pathways of complement-mediated lysis and shield other surface proteins from immune system. Thus, allowing them to persist in blood stream for extended periods (Pays and Nolan 1998). Furthermore, glycoproteins play a pivotal role in the life cycle, infection, and differentiation of protozoans (Niimi 2012). Bioinformatics and experimental methods have identified the general enzymatic machinery for synthesizing dolichol-linked precursors for *N*-linked oligosaccharides and the trafficking machinery for transferring them to nascent polypeptides (Luk et al. 2008; Samuelson et al. 2005). The protein-trafficking mechanism in Trypanosomatidae is quite similar to higher eukaryotes such as, mammals and yeasts. Like other eukaryotes, oligosaccharides are assembled on dolichol lipid in the ER, followed by transfer to the nascent polypeptide in the lumen of ER. After entering Golgi, oligosaccharides are modified by Golgi localized enzymes (Parodi 1993). Together with glycosylation potential, characteristic features such as, RNA editing, arrangement of genes in tandem arrays, polycistronic RNAs and trans-splicing, and regulation of gene expression at PTMs level make protozoan parasites suitable expression platform (Haile and Papadopoulou 2007; Liang et al. 2003; SIMPSON et al. 2003).

Leishmania tarentolae infecting lizard has been patented for producing recombinant proteins with homogenous *N*-glycans (Alexandrov and Grün 2001). LEXSY is an S1-classifed unicellular eukaryotic organism. Ease of handling, growth to high cell densities in cost-effective medium along with protein folding and PTMs machinery make LEXSY a promising expression candidate. Heterologous expression is carried out by integrating the gene in the *ssu* locus of chromosome via homologous recombination. Additionally, recently developed linear episomes provide the opportunity to propagate target gene in *L. tarentolae* for 90 generations without any major alterations in sequence or expression level (Kushnir et al. 2011). Publically available genome sequence of *L. tarentolae* allows to identify glycogenes and humanize the glycosylation pathways for the production of therapeutic glycoproteins (Raymond et al. 2011).

A small number of recombinant proteins including, bone morphogenetic proteins (BMPs), EPO, Laminin-332, and soluble amyloid precursor protein alpha (sAPPalpha) have been produced in LEXSY (Breitling et al. 2002; Klatt et al. 2013; Phan et al. 2009; Rahmati et al. 2016). LEXSY was able to secrete natively processed EPO with fully galactosylated and α1,6-fucosylated *N*-glycans, which exerted biological activity similar to that of its counterpart synthesized in CHO cells (Breitling et al. 2002). Heavily α1,6-fucosylated *N*-glycans of mAbs reduce ADCC. So it can be eliminated by inactivating the relevant gene (Sun et al. 2015; Yang et al. 2015). Only sialylation of *N*-glycans was missing, which could be achieved by using in vitro procedures or expressing the trans-sialidase of *T. cruzi* in *Leishmania* cells. The *N*-glycosylation of EPO was homogeneous with a mammalian-like biantennary oligosaccharide and the Man3GlcAc2 core structure. Homogenous *N*-glycan repertoire produced by LEXSY is important in cases where the recombinant protein needs to be crystalized for structural studies. Because small differences in molecular weight and charge among glycoforms make the isolation of homogenous *N*-glycans from human challenging (Breitling et al. 2002).

Laminin-332 is a heterotrimeric protein of α3-β3-γ2 subunits containing several cysteine residues that must be folded properly through intra-chain disulfide bonds. The LEXSY platform has been evaluated for the expression of this heterotrimeric protein (Phan et al. 2009). Correctly folded and assembled recombinant laminin-332 was purified from the culture medium. All three subunits were confirmed by immunoprecipitation and immunoblotting. Cell adhesion activity of eluted laminin-332 was similar to the analog produced in mammalian cells. This suggests that appropriate molecular chaperones for folding and a trafficking system for large proteins are present in LEXSY. Apart from *N*-glycosylation, LEXSY has the potential to perform initial steps of *O*-glycosylation. A recombinant form of sAPPalpha produced in *L. tarentolae* revealed *N*- and *O*-glycans on the same sites as described for its analog expressed in mammalian cells, and demonstrated similar biological activity (Klatt et al. 2013). However, larger *O*-glycans commonly present in mammalian cells were found missing in LEXSY-synthesized sAPPalpha.

Besides glycosylation potential and rapid growth to high cell densities, product can be obtained by disrupting the cells either by mild detergents and/or sonication. *L. tarentolae* cells grow on chemically defined media thereby, reducing the chance of contamination of recombinant proteins with prions or pathogenic viruses. Further studies analyzing the glycosylation pattern of glycoproteins and application of established glycoengineering techniques will make this system an alternate to CHO cells for producing cost effective recombinant proteins for therapeutic applications and structural studies.

Table 1 Humanizing the glycosylation profile of eukaryotic expression systems either by knocking out genes, encoding epitopes and/or expressing human glycogenes

Expression system	Glycoprotein expressed	Type of glycoengineering	References
S. cerevisiae	mAb	Humanizing mannose structure of *N*-glycans	Nasab et al. (2013)
P. pastoris	mAb	Uniform *N*-linked glycans of the type Man5GlcNAc2	Potgieter et al. (2009)
P. pastoris	rEPO	Fully complex terminally sialylated *N*-glycans	Hamilton et al. (2006)
P. pastoris	mAb	Improving the *N*-glycan site occupancy	Choi et al. (2012)
H. polymorpha	Recombinant glycoprotein	To attain human hybrid-type *N*-glycans with a terminal *N*-acetylglucosamine	Cheon et al. (2012)
S. frugiperda	rEPO	Humanizing sialylation	Mabashi-Asazuma et al. (2013)
Drosophila melanogaster	Cellular glycoproteins	Elimination of paucimannosidic residues and elongation of humanized *N*-glycans	Mabashi-Asazuma et al. (2015)
Insect cell lines	Rituximab	Elimination of core α1,3-fucosylation	Mabashi-Asazuma et al. (2014)
Insect cell line	Model glycoprotein	Humanizing glycosylation pathway	Aumiller et al. (2012)
Insect cell line	mAb	Humanizing complex *N*-glycans	Park et al. (2014)
CHO	rEPO	Enhancing *N*-glycan branching and sialylation	Yin et al. (2015)
CHO	Anti-CD2 IgG1	Improving ADCC of IgG1 by Fc-glycoengineering	Xu et al. (2016)
CHO	Recombinant glycoprotein	Elimination of Neu5Gc and αGal epitopes	Lin et al. (2015a)
CHO	IgG	Increasing sialylation	Lin et al. (2015b)
CHO	mAb	Production of defucosylated mAb	Sun et al. (2015)
Oriza staiva	Recombinant human granulocyte/macrophage colony-stimulating factor (hGM-CSF)	Elimination of α-1,3-fucose and β-1,2-xylose residues	Shin et al. (2011)
N. benthamiana	Human glucocerebrosidase	Achieving *N*-glycans with high mannose but devoid of α-1,3-fucose and β-1,2-xylose residues	Limkul et al. (2016)
N. benthamiana	Human mAb	*In vivo* sialylation	Castilho et al. (2010)
L. minor	Human mAb	Elimination of α-1,3-fucose and β-1,2-xylose	Cox et al. (2006)
P. patens	Anti-tumor antigen antibody (IGN311)	Elimination of α-1,3-fucose and β-1,2-xylose	Schuster et al. (2007)
L. tarentolae	Recombinant influenza haemagglutinins	No engineering, production of glycosylated and immunogenic vaccine	Pion et al. (2014)
L. tarentolae	rEPO	No engineering, production of fully galactosylated and α1,6-fucosylated rEPO	Breitling et al. (2002)
L. tarentolae	Laminin-332	No engineering, production of heterotrimeric glycoprotein	Phan et al. (2009)
L. tarentolae	sAPPalpha	No engineering, production of glycosylated sAPPalpha	Klatt et al. (2013)
L. tarentolae	Small surface antigen of hepatitis B virus	No engineering, production of chimeric glycoprotein	Czarnota et al. (2016)

Discussion

Despite rapid advances in expression technology and genetic engineering for producing therapeutic recombinant proteins (Khan and Sadroddiny 2015), hurdles remain to be resolved. Numerous glycosyltransferases embedded in ER and Golgi membrane have been identified in human, synthesizing a highly regulated repertoire of glycans (Breton et al. 2012). Glycans are multifunction structures, playing a vital role in cell adhesion, molecular trafficking, clearance from circulation, receptor activation, signal transduction, regulating immune system, and endocytosis. Several mutations in glycosyltransferases that lead to congenital disorders of glycosylation have been identified, highlighting the critical role of glycan structures in human development and physiology (Hennet and Cabalzar 2015).

The nature of *N*-glycans is determined by the type of protein and the cell in which they are produced. In addition,

glycosylation profile varies significantly across organisms. Different species produce different types of *N*-glycans repertoire. Generally, the process of glycosylation starts in the ER. The majority of the glycosylation steps are accomplished by Golgi-localized glycosyltransferases that subsequently generate mature glycan structures. In biotechnology industry, the glycosylation profile of the expression host is of utmost importance. Because glycans drive the biological activity, efficacy, immunogenicity, and serum half-life of therapeutic glycoproteins. Deep knowledge of glycosylation pathways and the consequences of their inactivation at any point are vital for devising glycoengineering strategies and synthesizing human-like glycans (Stanley 2011). Nevertheless, our understanding of glycan structures among eukaryotes is limited, which could be attributed to the non-template based biosynthesis, intricate glycosylation pathways, and the numerous enzymes modulating these pathways (Castilho et al. 2010).

Together with fine tuning the glycosylation machinery of conventional expression systems (Table 1), it is important to discover innovative expression hosts for producing inexpensive therapeutic glycoproteins. Evaluating a novel expression host requires considerations such as the endogenous glycosylation patterns as well as parameters including, the time required from exogenous gene integration to recombinant protein purification, the cost of production and purification, and the overall royalties associated with recombinant protein production. In this context, LEXSY is advantageous because it provides the opportunity to produce correctly folded and biologically active recombinant glycoproteins with mammalian-like *N*- and *O*-glycans (Klatt et al. 2013). A potentially beneficial feature of *N*-glycan repertoire is its homogeneity, which is highly demanded for structural studies of recombinant proteins and studying the consequences of *N*-glycans in vivo. LEXSY derived recombinant EPO revealed that *N*-glycans were fully galactosylated and 1,6-fucosylated. However, sialylation and higher branched, tri- and tetra-antennary glycans were not observed (Breitling et al. 2002). The latter one could attributed to the lack of *N*-acetylglucosaminyl transferase IV (Niimi 2012). Furthermore, no larger *O*-glycans were observed commonly present in mammalian expression systems (Klatt et al. 2013). Despite biotechnological importance, little attention has been paid to LEXSY in expression technology. On the base of these limited studies, one cannot completely rule out the expression potential of LEXSY. Further studies are needed to investigate the degree of suitability of LEXSY in biotechnology industry. To date, no glycoengineering strategies have been reported in LEXSY. By employing genetic engineering strategies, it is possible to produce sialylated glycoproteins with higher *N*- and *O*-glycan structures. Considering the benefits i.e. simplicity, ease of handling, and the potential of mammalian-like glycosylation, there is an urgent need to launch glycoengineering strategies in LEXSY. Even slight modifications such as, tailoring sialylation and larger *N*- and *O*-glycans reactions could make LEXSY an alternative expression platform particularly, when the cost of recombinant proteins is a major concern.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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