CRITICAL REVIEWS IN BIOTECHNOLOGY
2019, VOL. 39, NO. 3, 380–394
https://doi.org/10.1080/07388551.2019.1566206

Taylor & Francis
Taylor & Francis Group

REVIEW ARTICLE

Potential application of *Leishmania tarentolae* as an alternative platform for antibody expression

Jing Yi Lai[a], Stephan Klatt[b] and Theam Soon Lim[a,c]

[a]Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Penang, Malaysia; [b]The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, Victoria, Australia; [c]Analytical Biochemistry Research Centre, Universiti Sains Malaysia, Penang, Malaysia

ABSTRACT

Through the discovery of monoclonal antibody (mAb) technology, profound successes in medical treatment against a wide range of diseases have been achieved. This has led antibodies to emerge as a new class of biodrugs. As the "rising star" in the pharmaceutical market, extensive research and development in antibody production has been carried out in various expression systems including bacteria, insects, plants, yeasts, and mammalian cell lines. The major benefit of eukaryotic expression systems is the ability to carry out posttranslational modifications of the antibody. Glycosylation of therapeutic antibodies is one of these important modifications, due to its influence on antibody structure, stability, serum half-life, and complement recruitment. In recent years, the protozoan parasite *Leishmania tarentolae* has been introduced as a new eukaryotic expression system. *L. tarentolae* is rich in glycoproteins with oligosaccharide structures that are very similar to humans. Therefore, it is touted as a potential alternative to mammalian expression systems for therapeutic antibody production. Here, we present a comparative review on the features of the *L. tarentolae* expression system with other expression platforms such as bacteria, insect cells, yeasts, transgenic plants, and mammalian cells with a focus on mAb production.

ARTICLE HISTORY
Received 24 September 2018
Revised 29 October 2018
Accepted 9 November 2018

KEYWORDS
Leishmania tarentolae; protozoan; posttranslational modification; eukaryotic expression system; glycosylation; monoclonal antibodies

Introduction

Antibodies are useful tools for many major research and developmental activities including, but not limited to, biomarker discovery, disease diagnosis and therapy. Antibodies have gained a broad interest in today's biopharmaceutical market which has resulted in intensified research activities on antibody therapeutics to design better solutions for cancer, autoimmune diseases, and emerging infectious diseases [1–3]. High-throughput discovery of recombinant human monoclonal antibodies (mAbs) has been achieved through phage display technology in the past decade [4]. The introduction of semiautomated magnetic-based [5,6] and tip-based [7] biopanning procedures has helped to ease and speed the process of mAbs selection. However, the pace of mAb discovery suffers from the downstream production bottleneck when bulk production of pure and soluble recombinant mAbs in industrial scale is required. This shortcoming has highlighted the urgency to rapidly develop an efficient platform for mAb production. Until recently, 50% of therapeutic antibodies were mainly produced from mammalian cell lines, such as Chinese Hamster Ovarian (CHO), due to their close similarity to human posttranslational modification (PTM) patterns [8]. Exploration of alternative expression platforms such as bacteria [9], baculovirus (insects) [10], yeasts [11], transgenic plants [12], and cell-free expression systems [13] has also been carried out. Even so, utilization of these nonmammalian platforms is limited by their PTM and often requires additional humanization steps to increase the suitability of the mAbs for therapy.

Recently, the eukaryotic protozoan parasite *Leishmania tarentolae* has gained considerable interest as an alternative platform for mAb expression. *L. tarentolae* belongs to the class of Kinetoplastida [14] and to the order of Trypanosomatida [15]. The life cycle of *L. tarentolae* is complex and digenetic, involving two hosts – the sand fly (*Sergentomyia* spp.) as the insect vector [16,17] and gecko (*Tarentola mauritanica* and *Tarentola annularis*) as the vertebrate host [18,19]. In brief, *L. tarentolae* is assimilated by female sand flies during blood ingestion and develops extracellularly into flagellated promastigotes in the hindgut of sand flies [16]. The promastigotes will proliferate and migrate

CONTACT Theam Soon Lim theamsoon@usm.my Institute for Research in Molecular Medicine, Universiti Sains Malaysia, 11800 Penang, Malaysia

anteriorly to the proboscis (mouthpart) and are then introduced into the gecko when the sand fly bites or through ingestion of the infected sand fly [20]. Unlike other *Leishmania* that form intracellular amastigotes in their vertebrate host, *L. tarentolae* is rarely observed in the amastigotes stage. Instead it exists predominantly as promastigotes in the bloodstream and the lumen of the cloacae and intestine of gecko [21].

Several modes of recombinant protein expression are available for *L. tarentolae*, including secretory or intracellular expression through constitutive (genome integrated), inducible (genome integrated or episomal) or cell-free (plasmid-based or PCR-based) expression systems. The constitutive expression system maximizes the expression rate through integration of DNA into the 18S rRNA gene locus of *L. tarentolae* [22], whereas the inducible expression system provides tight control over the transcriptional machinery through the T7 RNA polymerase and TET repressor protein [23]. The cell free system provides rapid and flexible solutions to recombinant protein production [24]. Vector systems, depending on the presence of a signal peptide, can facilitate either secretory or intracellular protein expression [25]. In general, *L. tarentolae* is relatively easy and cheap to cultivate at high cell densities, offering beneficial potential to harvest recombinant proteins in the high-mg range [22,23,26]. In addition, *Leishmania* offers a unique transcription and translation machinery, which is valuable for molecular and cellular research as demonstrated in studies on telomere organization and glycosylphosphatidylinositol (GPI) anchoring of proteins [27,28]. In *Leishmania*, gene regulation is carried out at the posttranscriptional level through unique mechanisms such as polycistronic transcription, RNA editing, tandem array arrangements and trans-splicing [15,29,30]. Moreover, *Leishmania* is rich in glycoproteins capable of providing N-glycosylation (asparagine-linked) and initial O-glycosylation that are similar to mammalian cells and homogenous [22,31]. Glycosylation is one of the most important protein modifications as more than 50% of all human proteins are glycosylated [32], and recombinant glycoproteins are the most approved therapeutic proteins [33]. In addition, protein glycosylation is a quality control mechanism for the folding status of proteins [34], which increases their half-life and regulates protein interaction with cell receptors. In the latter case, the specific glycan composition or rather the terminal sugar residue directly regulates the immune response. Hence, the glycoprofile is very important for the stability, solubility, immunogenicity and serum half-life of mAbs, suggesting that *L. tarentolae* is a suitable host to produce recombinant antibodies that satisfies therapeutic requirements [35,36].

The scope of this review encompasses a comparative view point of the different systems used mainly for recombinant antibody production which includes bacteria, insect cell lines, yeast, transgenic plants, mammalian cell lines, and cell-free expression systems. The application of the different systems is then compared to that of *Leishmania tarentolae* while highlighting the advantages and disadvantages for glycosylated mAb production for improved therapeutic efficacy.

Recombinant antibody production

After the establishment of recombinant mAbs as a new class of therapeutic biodrugs, more than 40 mAbs have been marketed to treat different cancers and autoimmune diseases [37]. The application of mAbs for therapy is mainly attributed to their low toxicity, high specificity towards target antigens and the ability to induce natural immune responses such as the induction of apoptosis, opsonization, neutralization of soluble molecules responsible for diseases and functions as an agonists or antagonists of cellular activity [38].

There are five classes of immunoglobulins (Ig) namely; alpha (IgA), delta (IgD), gamma (IgG), mu (IgM), and epsilon (IgE). Of the five Ig isotypes present in mammals, IgG is the predominant isotype found in human and mouse serum [38]. Most of the currently licensed mAbs belong to the IgG isotype [39]. A deeper examination of the IgG isotype will show further classification into four subtypes (IgG1, IgG2, IgG3, and IgG4), each differentiated by the amino acid sequence variation in the constant region of the heavy chain. Structurally, IgG is a Y-shaped molecule comprising of two light chains and two heavy chains, each of which has repeating structural motifs that form into domains. The domains on the light chain and heavy chain will pair to interact covalently and non-covalently to form three moieties – two identical Fab (antigen-binding fragment) and one Fc (crystallizable fragment) (Figure 1(a)) [40–43]. Fab recognizes and binds to specific antigens, whereas the Fc moiety interacts with ligands such as Fc receptors (FcγRI, FcγRII, FcγRIII), C1q component of complement and neonatal Fc receptor (FcRn) [44]. Binding of Fc to FcRn assists transportation of IgG across the placenta and into the extravascular space. The interaction determines the catabolic half-life of antibodies and is independent of glycosylation [45]. On the other hand, binding of the Fc moiety to the FcγR and C1q complement elicits effector mechanisms such as antibody-dependent cellular cytotoxicity (ADCC) and

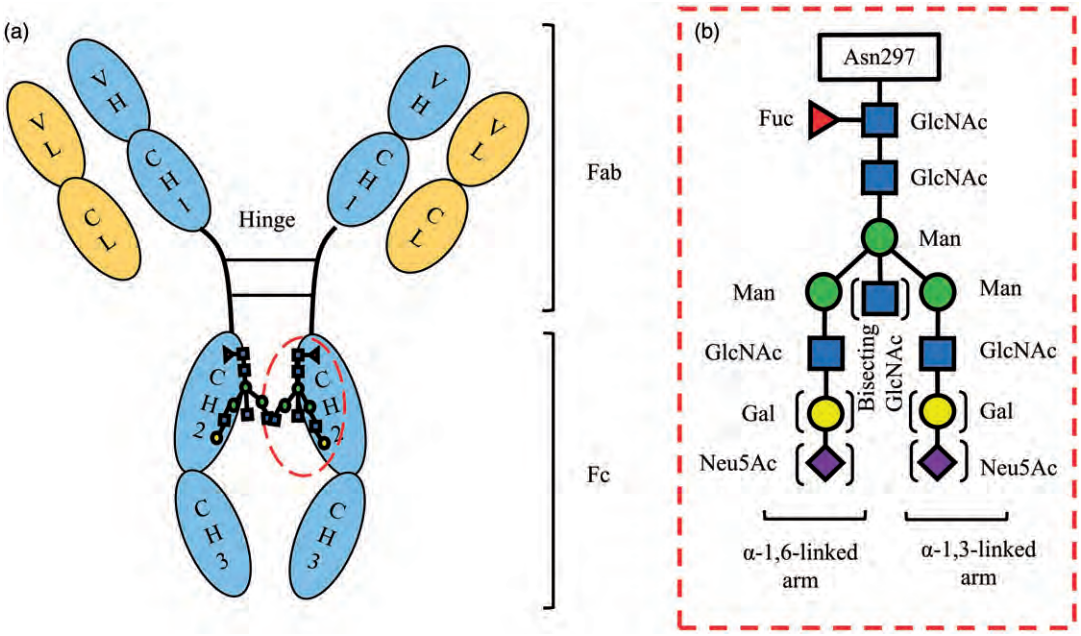

Figure 1. (a) Basic structure of immunoglobulin G (IgG). IgG contains two light chains and two heavy chains, paired into three moieties (two Fab and one Fc) and linked together through a flexible hinge region. Glycosylation occurs at a single site – Asn297 on C_H2 domain. The *N*-glycan (circled in dotted line) interacts with the surface of C_H2 and the opposing *N*-glycan to form a homodimer and horseshoe assembly. (b) N-linked glycan in human IgG Fc. The glycan attached to Asn297 in C_H2 domain has a basic octasaccharides core structure of $FucGlcNAc_2Man_3GlcNAc_2$ and may be extended by variable addition of galactose and sialic acid (indicated in bracket). The sugar branches at the central mannose into two arms: α-1,6-linked arm and α-1,3-linked arm. α-1,6-linked arm interacts with amino acid on C_H2 whereas α-1,3-linked arm interacts with the α-1,3-linked arm of opposing *N*-glycan.

complement-dependent cytotoxicity (CDC). The process is reliant on the asparagine-linked (N-linked) glycosylation of the Fc moiety [46–48]. Each IgG subtype demonstrates a unique profile of effector functions because of the variation in their binding ability to the Fcγ receptors and C1q complement [49–51]. Therefore, the choice of IgG subtype and the glycoprofile becomes a critical consideration when developing therapeutic mAbs.

In addition, production of therapeutic mAbs requires stringent regulation because therapeutic use of a non-native antibody structure with minor changes in its disulfide bonds and oligosaccharide structures can lead to adverse effects, like hypersensitivity, that adds further risk to the patients [52]. Moreover, both physical and chemical stability is another important issue in the development of therapeutic mAbs. Physical instability tends to cause formation of aggregates, whereas chemical instability tends to cause deamination or oxidation, which in both cases reduces the efficacy of mAbs as a therapeutic drug [53]. The stability of mAbs is proven to be associated with disulfide bonds and glycosylation within the mAbs structure [53,54].

In today's market, most of the commercially available mAbs were manufactured using mammalian cell lines with human-like glycoprofiles such as CHO, murine myeloma lymphoblastoid cells (NS0) and mouse Sp2/0 cell lines [38,39]. A bacterial host is not a usual choice for antibody production because the prokaryotic machinery is unable to carry out PTMs. Moreover, protein misfolding in bacteria often results in poor constructs that accumulate into nonfunctional inclusion bodies [55,56]. In short, careful selection of the production platform plus glycoengineering of mAbs to improve their clinical usage would be welcome improvement in mAbs development procedures.

Glycosylation of antibodies

Glycosylation is a unique PTM process that occurs in both the endoplasmic reticulum (ER) and Golgi apparatus, where a range of glycosidases and glycosyltransferases covalently conjugate oligosaccharides to an amino acid within a polypeptide chain [57–59]. Glycosylation in the ER is highly conserved in eukaryotes to yield a core heptasaccharide backbone, whereas glycosylation in the Golgi apparatus adds further sugar moieties in which the monosaccharide residues are species and cell line dependent [59,60]. Random combinations of the monosaccharide residues can generate hundreds of unique glycoprofiles [53]. While there are several types of glycosylation – O-linked, N-linked, C-linked, phosphoglycosylation, and

glypiation [61], the two main types of glycosylation in antibodies are O-linked and N-linked glycosylation [62]. O-linked glycosylation occurs by attachment of *N*-acetylgalactosamine (GalNAc) to the oxygen atom of serine (Ser), threonine (Thr), or tyrosine (Tyr) residues. N-linked glycosylation involves the attachment of *N*-acetylglucosamine (GlcNAc) to a nitrogen atom of asparagine (Asn) with the defined sequence Asn-X-Thr/Ser-Y, where X and Y can be any amino acid except proline [40,63].

In human IgG Fc, the glycosylation is N-linked with complex biantennary type glycans, containing a core heptasaccharides with variable outer arm monosaccharide residues, such as fucose (Fuc), galactose (Gal), bisecting GlcNAc (i.e. GlcNAc that is added to the base mannose residue of the trimannosyl core) and sialic acid (Neu5Ac). The main *N*-glycan structure observed on human IgG C_H2 domain (specifically on Asn297) is an octasaccharide with a fucose, four *N*-acetylglucosamine and three mannose residues ($FucGlcNAc_2Man_3GlcNAc_2$). This may be extended by the addition of galactose and sialic acid (Figure 1(b)). The glycan branches at the central mannose into two arms: α-1,6-linked arm interacts non-covalently with the protein surface, particularly hydrophobic amino acids on the C_H2 domain, whereas α-1,3-linked arm interacts with α-1,3-linked arm of *N*-glycan from opposing C_H2 domain to form homodimers and maintain the horseshoe-like assembly of the Fc (Figure 1) [64,65]. Glycosylation at the C_H2 domain has no effect on the antigen-binding capability. Instead, oligosaccharides are shown to influence the Fc-mediated effector functions of the antibody and the action efficacy is mediated by the type of oligosaccharide [66].

The variations in the IgG glycosylation pattern are known to alter the efficacy of Fc binding to the FcγR that sensitizes the target for downstream ADCC, CDC and/or apoptosis [40]. Analysis of rituximab, the first anti-CD20 designed for the treatment of cancer and autoimmune diseases, suggests that engagement of CD20-bound Fc to FcγRIIIa on natural killer (NK) cells in the ADCC mechanism is dependent on the fucosylation status. An afucosylated rituximab-like product was shown to have enhanced ADCC activities, largely due to the reduced steric hindrance upon interaction with the highly glycosylated FcγRIII receptor [67]. Similarly, analysis of trastuzumab suggests that a lack of fucose in the Fc could improve the ADCC mechanism [68]. Glycosylation profile engineering could improve sensitization of mAbs and is useful for the development of mAbs against diseases that produce low levels of antigens, such as lymphoma and leukemia [36].

The glycoprofile also influences the solubility, stability and half-life of the antibodies, which are also critical aspects to consider in therapeutic antibody development [53]. Aglycosylated or deglycosylated IgG has been shown to have reduced functionality and stability due to the absence of masking effects provided by the *N*-glycan [60,61]. While α-1,6-linked arm of *N*-glycan interacts with the hydrophobic amino acids on the C_H2 domain, it shields the hydrophobic region of the Fc from the solvent [69]. Thus, changes or removal of the glycan structures will alter the hydrophobicity of antibodies that will eventually lead to the formation of aggregates [53].

Different organisms and species used for mAb expression will yield unique glycoprofiles that give different therapeutic efficacies. Alemtuzumab generated in the rat YB2/0 cell line was found to present higher efficacy as opposed to those produced from mouse NS0 and hamster CHO cell lines [70]. This highlights that the choice of the production platform has been a major consideration in generating proper glycosylated therapeutic mAbs [71]. Moreover, the addition of monosaccharides that are not naturally found in human serum can influence the immunogenicity of the mAbs. For example, the addition of galactose in an α-1,3-linkage, *N*-acetylneuraminic acid (Neu5Ac) in an α-2,3-linkage and *N*-glycolylneuraminic acid (Neu5Gc) which does not naturally occur in humans, are commonly introduced by mouse NS0, hamster CHO and mouse Sp2/0 cell lines. These glycans are immunogenic to humans when administered [72,73]. This is true in the case of cetuximab produced from mouse Sp2/0 cells, that contains both α-1,3-galactose and α-2,3-linked Neu5Gc, and caused an increase in hypersensitivity in 33% of the patients receiving cetuximab treatment [74,75].

Glycosylation is very important in determining the quality of therapeutic mAbs. Production of mAbs with the "correct" and homogenous glycoforms to maximize the therapeutic efficacy has become a major challenge in biopharmaceutical research as the glycoprofile in nonhuman related platforms often differs significantly from human glycoprofiles. Expression platforms such as bacteria do not perform human protein glycosylation, while other eukaryotic expression hosts like yeasts, plants, insects, and nonhuman mammalian cells produce glycans that are different from humans [76] (Figure 2). To accommodate these needs, knockout and knock-in cell lines originating from hamster CHO, *Pichia pastoris,* and *Lemna* have been generated to produce non-fucosylated, non-galactosylated, fully galactosylated, and fully sialylated mAbs [38]. In addition to

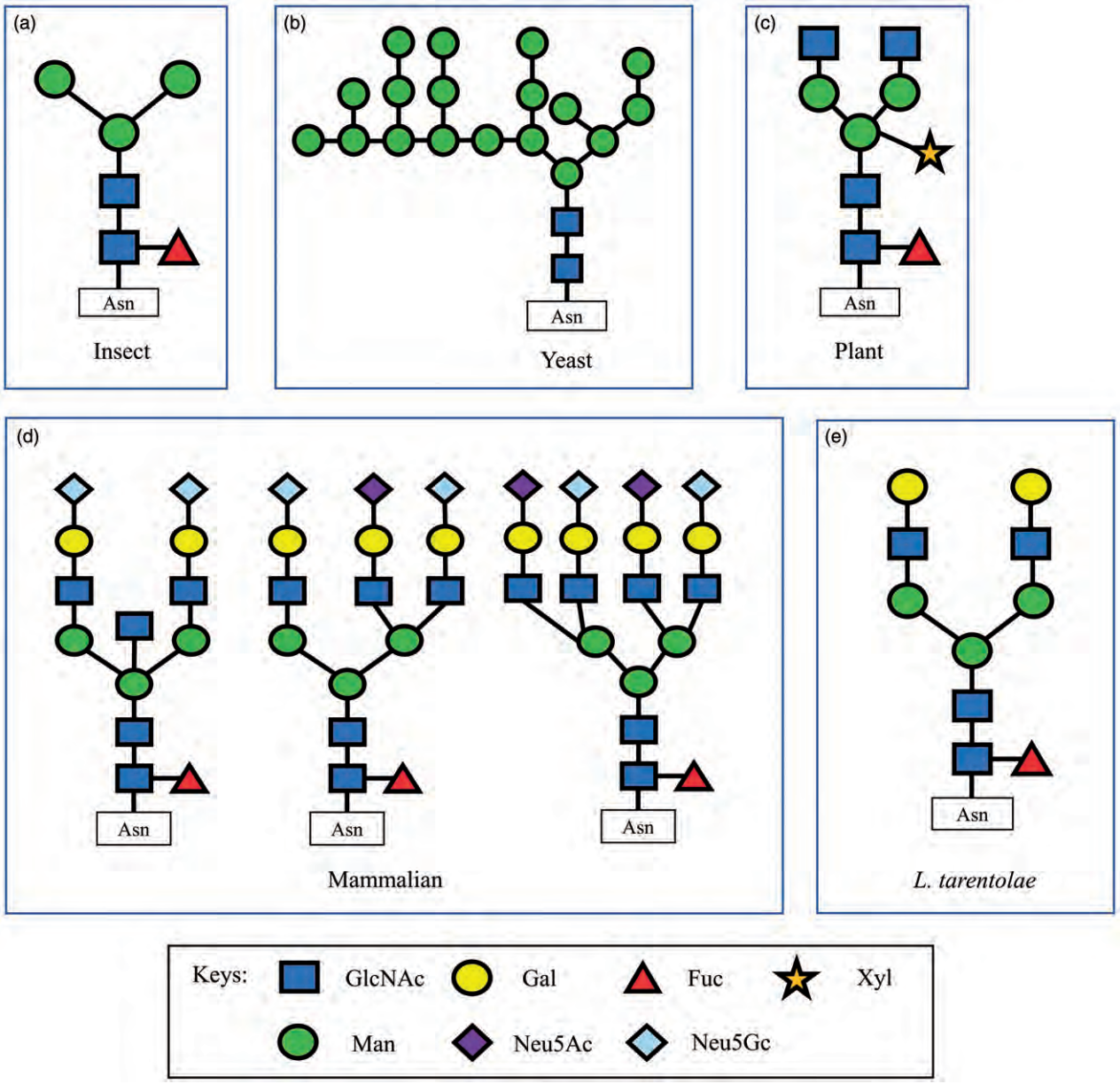

Figure 2. N-linked glycosylation pattern derived from different hosts. (a) *Insect*: insect cell produces simple paucimannose *N*-glycan without complex side chains. (b) *Yeast*: *N*-glycan in yeast is highly branched and mannose rich. (c) *Plant*: *N*-glycan in plant has β-1,2-xylose which is plant-specific and immunogenic to human. (d) *Mammalian cell lines*: mammalian *N*-glycan is similar to humans, consisting complex bi-, tri-, and tetra-antennary structures. The structure comprises of a core heptasaccharides, two mannose arms and the addition of fucose (Fuc), galactose (Gal), bisecting *N*-acetylglucosamine (GlcNAc) (i.e. GlcNAc attached to base mannose of trimannosyl core), *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) in different combination, depending on the cell line and cell culture conditions. (e) *L. tarentolae*: glycosylation in *L. tarentolae* is highly similar to mammalian-type N-glycosylation but is devoid of the terminal sialic acid.

these expanding efforts to humanize different hosts, many researchers are also venturing into the development of new expression platforms. A suggested new platform for the expression of therapeutic antibodies is *Leishmania tarentolae*. It is capable of producing glycoproteins which include α-linked galactose and fucose residues leading to proteins that are highly similar to the human counterpart [77].

Conventional antibody expression systems

Bacteria

Bacterial systems have been widely utilized as the workhorse for protein expression in the past few decades. The most notable bacterial host is *Escherichia coli* (*E. coli*), a Gram-negative bacterium that survives in the intestine of many vertebrates. This is the preferred

expression host for most of the recombinant proteins, due to its rapid growth rate with a doubling time of 20 min, low cost in a cultivation medium and its capability to be easily transformed and maintained. Extensive studies on the genetic, biochemical and physiological characteristics of bacteria in general have led to the development of a wide range of genetic manipulation tools, well-established cloning vectors and various host strains for effective protein expression [78].

In spite of the advantages, bacterial systems also have several drawbacks that limit its suitability for mAb production. Improper protein folding, endotoxin contamination, amino acid substitution due to codon bias and accumulation of large proteins into insoluble, non-functional inclusion bodies are factors that limit the production of full-sized, functional and safe therapeutic mAbs from bacterial hosts. Bacteria do not perform PTM that is crucial to form biologically active and functional mAbs [79]. Glycosylation in *E. coli* has been attempted through the introduction of N-linked glycosylation cassettes [80,81], but is generally unsuitable due to the lack of the PTM machinery and organelles required for glycosylation.

Nonetheless, bacteria remain a popular choice to produce aglycosylated antibodies or antibodies in alternative formats such as single-chain fragment variables (scFvs) and fragment antigen binding (Fab), whose efficacy is not influenced by Fc glycosylation [26]. Even without the glycan, serum half-life and stability of bacteria-yield mAbs can still be improved. This can be achieved using modifications that involve conjugation with polyethylene glycol (PEG) to increase the serum half-life and stability [82]. It is this ability that has allowed for bacteria expression systems to remain relevant for mAb production.

Insect cell lines

Recombinant protein production in insect cell lines has been achieved through infection by insect-specific baculoviruses, commonly known as baculovirus expression systems. The commonly used insect cell lines include *Sf*-9, *Sf*-21 (derived from *Spodoptera frugiperda*) [83,84], S2 (derived from *Drosophila melanogaster*) and Hi-5 (derived from *Trichoplusia ni*) [85]. Manipulation of the insect cells involves homologous recombination [86] or DNA transposition [87]. The cell lines are maintained either in adherent or suspension cultures, without the need of CO_2 incubators. The cost of production using this system is relatively high. Baculovirus does not infect mammalian cells and are considered harmless to humans, making it useful for antibody production. Strong transcriptional promoters from baculoviral *polyhedrin* gene provides high yield of recombinant proteins – up to 300 mg/L [88].

Insect cell lines have been utilized for the production of several FDA-approved mAbs [82]. Although insect cells can carry out N-linked protein glycosylation, they are unable to produce sialylated *N*-glycans. Instead, they produce paucimannose glycans with α-1.3-fucose linkage that are insect-specific and allergenic to human [89,90] (Figure 2(a)). Humanization of insect-based mAbs can be achieved through suppression of β-*N*-acetylglucosaminidase, introduction of β-1,4-galactosyltransferase (GalT) [91] and α-2,6-sialyltransferase [92] to produce human-like terminally sialylated biantennary *N*-glycan.

Yeasts

Yeasts are eukaryotic unicellular fungi with rapid growth rates. They are cultured in low cost media. They are able to grow in high cell densities and can easily be scaled up to a bioreactor environment. *Pichia pastoris*, *Saccharomyces cerevisiae*, and *Pichia angusta* (formerly known as *Hansenula polymorpha*) are a few of the common yeast species used for glycoprotein production [93]. As eukaryotes, yeasts possess PTM machinery but their N-linked protein glycosylation profile is different from that of native human proteins. While the initial glycosylation process is very similar to humans where *N*-glycan is added to the serine or threonine residue with Asn-X-Ser/Thr-Y motif, further glycosylation is limited to mannose and mannosylphosphate. High mannosylation in antibodies reduces their half-life and may also induce immunogenicity in humans [94] (Figure 2(b)). Therefore, yeasts are mainly suitable for the expression of antibodies that do not require glycosylation.

Humanization of yeasts to produce human-like glycans has been attempted through knockout of the mannosyltransferase-encoding gene which consequently eliminates hyper-mannosylation while introduction of human glycosylation enzymes such as mannosidases I and II, *N*-acetylglucosaminyl transferases I and II, and uridine 5′-diphosphate (UDP)-*N*-acetylglucosamine transporter promotes production of the human *N*-glycan intermediate, $Man_5GlcNAc_2$ [95,96]. These humanization processes also increase the occurrence of a single glycoprofile (homogeneous glycan pattern) [97] and aid the optimization of the effector functions of the Fc [98]. This is well demonstrated in rituximab and its counterpart Rituxan. The efficacy of

humanized rituximab produced in various glycoengineered *P. pastoris* cell lines has shown 100-fold higher Fcγ binding affinity in comparison to Rituxan, a commercial counterpart produced in hamster CHO cells [99].

Transgenic plants

Expression of recombinant human proteins using plants is an interesting approach. The use of transgenic plants as an mAb production platform is not common and there is only a limited set of technologies available to efficiently manipulate the expression process in plants. However, this cross-kingdom vegetal system can be advantageous in terms of cost effectiveness and ease to scale up [8]. Hosts apart from the animal kingdom could lower the risk of pathogenic cross-contamination that affects humans [8]. The glycosylation process in plants is surprisingly similar to that of humans, except for the presence of plant-specific residues such as the core α-1,3-fucose and attachment of β-1,2-xylose to the core heptasaccharides, which makes the product potentially immunogenic [100] (Figure 2(c)). To generate pharmaceutical valuable mAbs, humanization of an mAb against CD30 in *Lemna minor* has been achieved through ribonucleic acid interference (RNAi) technology that interferes with α-1,3-fucosyltransferase and β-1,2-xylosyltransferase synthesis [101]. Terminal sialylation in plant expression systems has been tackled through the co-expression of the mammalian sialylation gene along with the mAb construct in, e.g. *Nicotiana benthamiana* [102]. These advances in plant glycoengineering allow the production of improved therapeutic mAbs. In fact, *N. benthamiana* has been employed to produce ZMapp, an antibody cocktail against Ebola virus, by the knockdown of the fucosyltransferase and the xylosyltransferase [12]. The glycoengineered *N. benthamiana* omitted the immunogenic nonhuman fucose and xylose, while the single glycoform production also greatly benefits the ADCC potency (ED_{50}=3 μg) as compared to the counterpart produced in mammalian hamster CHO cells (ED_{50}=11 μg) [103,104].

Mammalian cell lines

Mammalian cell lines would seem to be the ideal platform to produce recombinant human proteins. Commonly used mammalian cell lines include CHO, NS0 and human embryonic kidney 293 cells (HEK 293). Mammalian cell lines can yield 50 mg/L to 5 g/L of soluble protein with close-to-human glycosylation profiles. Nonhuman mammalian cell lines such as mouse NS0 and hamster CHO have glycosylation profiles slightly different from humans: sialic acid in α-2,3-linkages, the addition of α-1,3-galactose (α-Gal) that elicits adverse effects as demonstrated in Erbitux (cetuximab), addition of nonhuman *N*-glycolylneuraminic acid (Neu5Gc) and high amounts of α-1,6-fucose that could reduce therapeutic efficacy of the mAbs [36] (Figure 2(d)). Nonetheless, these drawbacks can be overcome through glycoengineering to promote defucosylation, increase sialylation, galactosylation, and mannosylation of the mAbs to improve their therapeutic efficacy [105].

Other drawbacks of mammalian cell lines include the risk of contaminations, especially viral or mycoplasmic contaminations from the serum supplements which could also infect humans. The growth of mammalian cells is relatively slow with a lower yield when compared to prokaryotic expression systems. The cultivation process is labor intensive and stringent requirements for laboratory operations have increased the costs, making it uneconomical for many productions. The pros and cons of bacteria, insects, yeast, and mammalian cell line expression systems are summarized in Table 1.

Cell-free expression systems

Another protein production platform for consideration is the cell-free protein expression system (CFES), in which it is carried out in an artificial environment supplemented with cell lysates, energy sources, and amino acid building blocks. Independent from the host cells, this *in vitro* system provides quick recombinant protein production that yields a product within few hours. Some commercially available cell lysates included those derived from *E. coli* [93], wheat germ [94], insect cells [106], yeast [107], rabbit reticulocytes [108], mouse CHO [109], and *L. tarentolae* [24]. The cost of CFES is higher, incurred by the necessity to supply nucleotides, amino acids, energy system and stabilizing compounds for the cell lysate to carry out protein production. However, the capability of CFES in antibody production should not be overlooked. Freedom from the boundaries of host cell mechanisms, offers CFES great flexibility in protein production. The system allows disulfide bond formation, proper folding and correct assembly of antibodies through supplementation with glutathione redox buffer, addition of exogenous protein disulfide isomerase or introduction of chaperonins like DnaK, DnaJ, GroEL, and GroES [95–98]. Through these supplements, prokaryotic *E. coli* lysates can successfully produce properly folded scFv, Fab, and even full-size IgG [13,99–103]. Cell-free protein production has been successfully carried out in other eukaryotes such as insect

Table 1. Comparison of different protein expression platforms.

	Bacteria	Insect	Yeast	Plant	Mammalian	*L. tarentolae*
Supplement for growth	Not necessary	Not necessary	Not necessary	Not necessary	Animal-based serum	Hemin
Costs	Very low	High	Low	Low	High	Low to moderate
Growth rate	Fast (division time 20–30 minutes)	Slow (division time 18–24 h)	Fast (division time 2–4 h)	Slow (division time 16 h)	Slow (division time 24 h)	Moderate (division time 6–9 h)
Up scaling	Easy	Fair, suitable for batch production only	Easy	Easy	Not easy	Fair
Protein yield	High	Low to high	Low to high	Low to high	Moderate to high	Moderate
Protein quality	Poor, formation of inclusion bodies, biased codon usage	Good, properly folded	Fair, truncated protein	Good, properly folded	Good, properly folded	Good, properly folded
Glycosylation	No	Nonmammalian type, lack of multiantennary glycans	High mannose, nonmammalian type	Nonmammalian type, contain xylose	Yes, highest similarity to humans	Mammalian-type (missing sialic acid only)
End products contamination risk	Endotoxins	Low	Low	Low	Prion, Virion	Low

cells, rabbit reticulocytes and wheat germ cells leading to functionally expressed antibodies [104,105]. While eukaryotic lysates retain the ability to carry out PTM in their endogenous microsomal vesicles [106,107], introduction of internal ribosome entry site (IRES) elements enables the production of glycosylated protein as exemplified in the production of erythropoietin using eukaryotic cell lysates [108]. Although there are no clinically approved mAbs produced currently from CFES, the system is another feasible platform for their production, especially with future advancements in glycoengineering.

Leishmania tarentolae as a potential mAb expression platform alternative

Leishmania tarentolae is a unicellular eukaryotic protozoan parasite and belongs to the class of Kinetoplastida [14] and to the order of Trypanosomatida [15]. It was originally isolated from the ringed wall gecko *Tarentola annularis* and *Tarentola mauritanica* in 1921 [18,19]. Its life cycle is characterized by alternating between sand flies (insect vector) and the gecko (vertebrate host), with the phenotypes of promastigotes and amastigote-like forms. Promastigotes are mono-flagellated lance-like structures with a length of 4–12 μm and a width of 0.5–3 μm which is the typical form found in cell culture. Amastigote-like cells are smaller and round in shape with a rudimentary flagellum. Many human pathogenic *Leishmania* species exist which cause different forms of leishmaniasis. However, *L. tarentolae* strain TARII is not pathogenic to humans. The genome of *L. tarentolae* has been fully sequenced, allowing a deeper understanding and recombinant engineering of this parasitic protozoan host [109].

In the past 25 years, this parasite has been utilized for a broad range of biotechnological and biomedical applications. In particular, its protein expression system is gaining more and more interest among researchers, because this parasite is easy to cultivate, exhibits eukaryotic PTM and can easily grow to high cell densities (up to 5×10^8 cells/mL) with the potential to harvest recombinant proteins in the high-mg range – up to 30 mg/L [16,110]. *L. tarentolae* is grown as static or agitated suspension culture in either a brain–heart infusion (BHI) medium or a yeast soybean based complex medium at 26 °C in the dark without the need of sera or a CO_2 incubator. Cultivation of cells in a bioreactor for bulk protein production for the industry is also well-established. Therefore, this system is relatively cheaper compared to mammalian cell cultures and it provides a good alternative. Growth media are usually supplemented with hemin, an iron-containing porphyrin, which enhances cell proliferation, regulates metabolic pathways of respiration and protein synthesis [111,112]. Besides the expensive BHI medium, Terrific broth with glycerol and glucose has been used for *L. tarentolae* cultivation [110,111], adding advantages in reducing the production cost to levels that are comparable with *E. coli*. The serum-free growth environment is also advantageous to prevent viral or prion contaminations, making products generally safe for consumption or biomedical applications. Moreover, *L. tarentolae* secretes very low levels of endogenous proteins, making it well suited for the expression of secretory recombinant proteins [77]. The application of *L. tarentolae* systems specifically for recombinant antibody expressions is listed in Table 2.

The uniqueness of the *L. tarentolae* expression system comes from its transcriptional and translational machinery. Briefly, the central dogma of molecular biology requires endogenous RNA polymerase (pol) II to drive the transcription of recombinant DNA cassettes and the process is well regulated. Overexpression of

Table 2. List of recombinant antibodies produced in *Leishmania tarentolae*.

Antibody format	Target	Antibody origin	Yield (mg/L)	Reference
scFv	–	Human recombinant scFv	0.04–3.83	[31]
scFv	Human vimentin	Human recombinant scFv	0.3–1	[112]
scFv-rFc		Human recombinant scFv, rabbit IgG Fc	1–2.95	
scFv	Murine laminin	Human recombinant scFv	0.3–1	
scFv-rFc		Human recombinant scFv, rabbit IgG Fc	1–2.95	
sdAb-rFc	Human pericyte fibronectin	Human synthetic domain, rabbit IgG Fc	–	[122]

recombinant proteins will trigger the transcriptional control mechanism in the host, which then downregulates the RNA pol II mediated transcription, resulting in low protein production. In *E. coli*, the downregulation can be bypassed through the introduction of T7 RNA polymerase (from the T7 bacteriophage). However, the same approach is not applicable to eukaryotic expression systems, due to the complexity of their transcription and translation mechanisms. In eukaryotes, pre-mRNA transcribed by pol II requires splicing, post-transcriptional RNA capping and polyadenylation (addition of poly(A) tail) to generate mature mRNA for effective translation (Figure 3). Since the capping occurs exclusively for RNA pol II-mediated transcription, employment of foreign RNA polymerases to bypass the gene regulation like *E. coli* is not feasible. *L. tarentolae* and other Trypanosomatida are interesting models with unusual mechanisms compared to other eukaryotes. In *L. tarentolae*, the endogenous RNA pol II transcribes genes into polycistronic pre-mRNA, which is then trans-spliced into individual mRNA for translation. A 39-nucleotide spliced leader is added at the 5′ end of the mRNA to form a cap during trans-splicing while the poly(A) tail is added through polyadenylation. The trans-splicing mechanism uncouples the transcription and translation mechanisms, allowing pre-mRNA to be transcribed by pol I and other foreign polymerases such as bacteriophage T3 and T7 polymerases [113]. The expression is regulated posttranscriptionally by intergenic untranslated regions (UTRs) or intergenic region (IR) flanking the open reading frame (ORF). Moreover, in *L. tarentolae* all the endogenous mRNAs have the same splice leader sequence. Therefore, in cell-free expression system, a single antisense oligonucleotide specifically targeting to the splice leader sequence can suppress all the endogenous protein expressions [110]. These unique characteristics expand the flexibility of *L. tarentolae* as an efficient protein expression platform [23,114]. Figure 4 shows the basic components required in an expression vector for *L. tarentolae*.

The expression efficiency and recombinant protein yields are largely dependent on the vector design. A simple vector should comprise of (A) the 5′ and 3′ intergenic UTRs, (B) the 5′ and 3′ small subunit rRNA locus (ssu; from *L. tarentolae*), (C) the antibiotic resistance gene, and, for secretory systems, a (D) signal peptide from the secreted acid phosphatase (sAP, from *L. mexicana*) (Figure 4). The choice of the UTRs is an important criterion to achieve high expression levels, as posttranscriptional gene regulation in *Leishmania* occurs through the UTRs. As reported by Breitling et al. [22], UTRs from the *L. tarentolae* calmodulin cluster, including three fragments and splice acceptor sites, drive the high protein expression levels. Within the vector, the gene-of-interest will be flanked by repetitive sequences of the ssu locus (5′ and 3′ end) to facilitate genomic integration into the ssu gene locus which is strongly transcribed by RNA pol I [115]. The integration ensures stable protein expression up to 90 parasite generations, without a loss or a change of the inserted DNA [116]. Transfection of *L. tarentolae* can be easily achieved through electroporation, and selection can be done with antibiotics [117]. Integration of multiple gene cassettes into the same mutant is also possible by using different antibiotic resistant genes, leading to the simultaneous expression of recombinant proteins to form multi-subunit proteins [118,119]. As an example, recombinant human laminin (LM)-332, a large heterotrimeric glycoprotein, could be successfully expressed in *L. tarentolae*. The trimer was efficiently formed through correct disulfide bond formation and was functional [119]. The possibility to express multi-subunit proteins in *L. tarentolae* is very advantageous as it allows the expression of full-length antibodies, which consist of the heavy and light chain subunits. Large molecular weight proteins (>100 kDa) have also been successfully expressed in *L. tarentolae* [116], supporting its suitability to produce full-length antibodies.

The major advantage of the *L. tarentolae* expression system is its ability to produce mammalian-type PTM, like phosphorylation and glycosylation. *L. tarentolae* has been shown to express mammalian-type biantennary *N*-glycans, with only the terminal sialic acids (*N*-acetylneuraminic acid) missing [22] (Figure 2(e)). *N*-glycan structures in *L. tarentolae* are highly homogenous. Higher branched glycans (tri- and tetra-antennary) are not described in *L. tarentolae*, probably due to the absence of *N*-acetylglucosaminyltransferase IV activity.

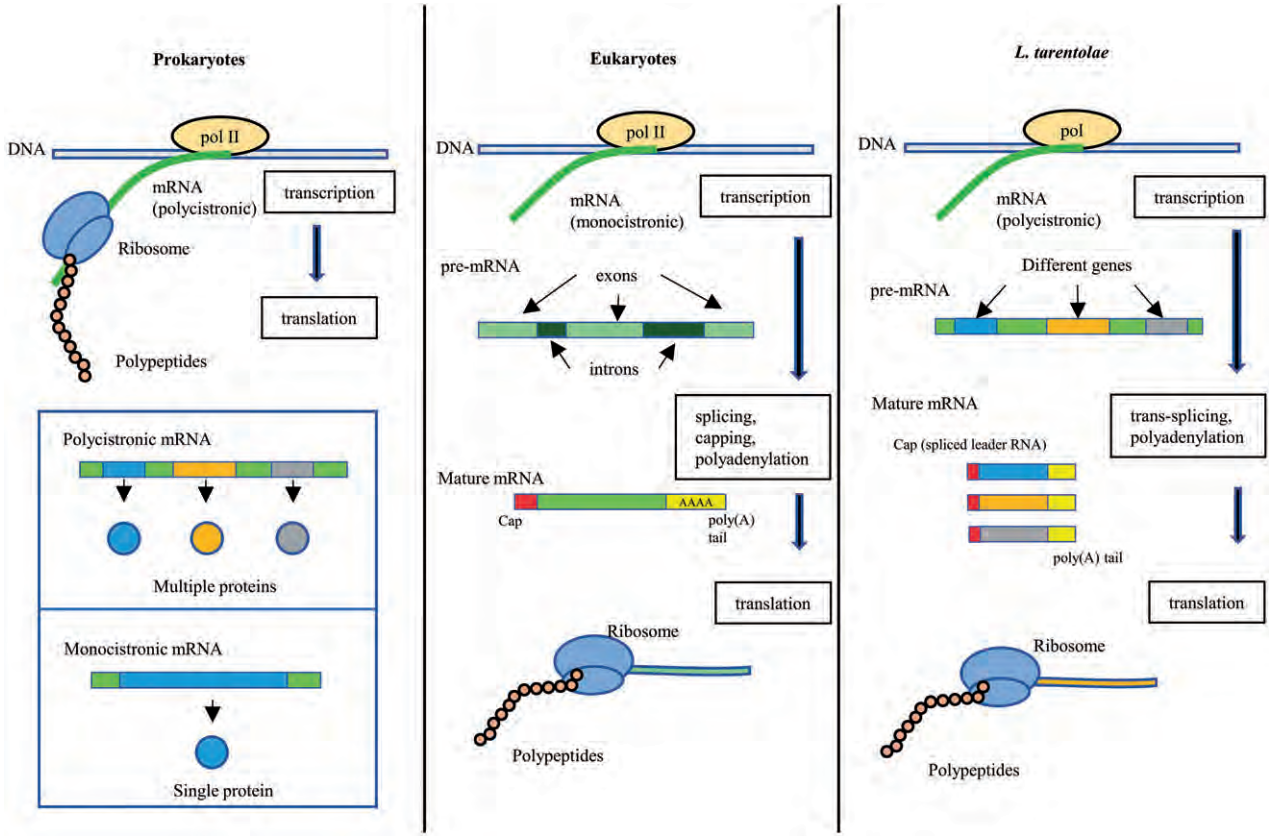

Figure 3. Schematic representation of coupled and uncoupled transcription–translation mechanism. In prokaryotes, the transcription and translation mechanisms are coupled, i.e. the mRNA is translated into protein immediately after it is transcribed by RNA polymerase II (pol II). Therefore, the gene regulation is simple and changes in either one of the process will directly affect the other process. In eukaryotes, the transcription and translation mechanisms are complex. Upon transcription, the pre-mRNA requires RNA processing such as splicing to remove noncoding introns, capping and polyadenylation to yield mature mRNA prior translation. The capping process occurs exclusively on pol II-mediated pre-mRNA. Consequently, the gene regulation mechanism in eukaryotes is more complicated. In prokaryotes, the mRNA may be polycistronic and encodes for multiple protein whereas in eukaryotes the mRNA is monocistronic and encodes for only one protein. In *L. tarentolae*, the transcription and translation mechanisms are different. As a eukaryote, *L. tarentolae* produces polycistronic mRNA which is then processed into individual mRNA through trans-splicing. A 39-nucleotide spliced leader is added as the 5′ cap during trans-splicing, poly(A) tail is added in polyadenylation to form a mature mRNA for translation. This trans-splicing mechanism uncouples the transcription and translation mechanisms as capping of pre-mRNA is not dependent specifically to pol II-mediated transcription.

The ability of *L. tarentolae* to produce *O*-glycosylate human recombinant proteins has also been shown with the expression of sAPPalpha, which plays a key role in Alzheimer's disease [120]. Full-length antibodies have not been expressed in *L. tarentolae* so far, but scFvs [31], scFv fused with Fc of rabbit IgG (scFv-rFc) [112] and single domain-rFc [121] have been reported. To date, there are no glycoengineered mAbs produced in *L. tarentolae* in the market, but with the availability of full genome sequence of *L. tarentolae*, strategies on genetic engineering to overcome missing sialylation, reduce fucosylation and produce more complex *N*-glycans and/or *O*-glycans would be feasible [21]. As recently suggested [36], integration of the trans-sialidase (TS) gene, an enzyme of Trypanosoma species, can help transfer sialic acids to endogenous glycoproteins and further humanize the *L. tarentolae* glycosylation profile [122,123]. The cell-free approach has also been established and tested in this parasite [110]. Although production of antibodies through *L. tarentolae* cell free expression system is not yet reported, there is huge potential to develop an alternative mAb production platform by merging the advantages of CFES rapid production with the *L. tarentolae* glycosylation profile.

Conclusions

The production of therapeutic mAbs is a multi-million-dollar industry [124], making them a popular molecule for development. Moving into the translational medicine era, bulk production of mAbs at low costs while maintaining proper folding and PTMs like glycosylation to ensure functionality efficacy are important to ensure availability of therapeutics to patients from all social

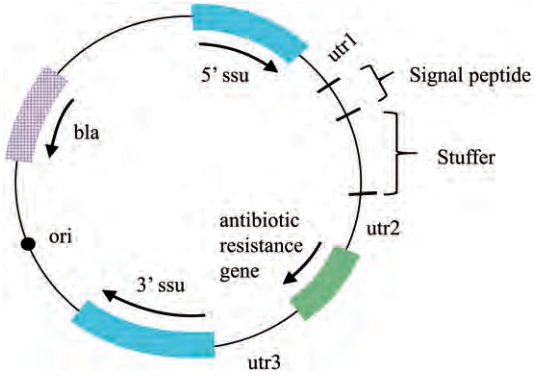

Figure 4. Schematic representation of a standard vector for *L. tarentolae* expression system. The basic vector system comprises of intergenic untranslated regions (utr1, utr2, utr3), 5′ and 3′ small subunit rRNA locus (ssu, from *L. tarentolae*), antibiotic resistance gene and signal peptide from the secreted acid phosphatase (sAP, from *L. mexicana*). bla: β-lactomase; ori: origin of replication from *E. coli*.

classes. Glycoengineering of expression platforms like bacteria, insects, yeasts, plants and mammalian cell lines improves the production of mAbs. On top of that, the new expression platform of *L. tarentolae* has beneficial characteristics over other conventional expression platforms with their eukaryotic PTM machinery being highly similar to human, and prokaryotic characteristic with high production yields. Although not established as a preferred system for antibody production, the prospect of utilizing such a system is intriguing and is attracting further investigation, especially in terms of immunogenicity. We foresee the potential application of *L. tarentolae* as an alternative expression system for mAb production in the near future.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The authors would like to acknowledge financial support from Universiti Sains Malaysia Research University Individual Grant Scheme [Grant No. 1001/CIPPM/812173] and the Malaysian Ministry of Education through the Higher Institution Centre of Excellence (HICoE) Grant [Grant No. 311/CIPPM/4401005]. JYL would like to acknowledge support from Graduate Assistant Scheme from Universiti Sains Malaysia.

ORCID

Jing Yi Lai http://orcid.org/0000-0001-5675-2998
Stephan Klatt http://orcid.org/0000-0003-0064-3367
Theam Soon Lim http://orcid.org/0000-0002-0656-3045

References

[1] Chan AC, Carter PJ. Therapeutic antibodies for autoimmunity and inflammation. Nat Rev Immunol. 2010; 10:301–316.
[2] Casadevall A, Dadachova E, Pirofski La. Passive antibody therapy for infectious diseases. Nat Rev Microbiol. 2004;2:695.
[3] Scott AM, Wolchok JD, Old LJ. Antibody therapy of cancer. Nat Rev Cancer. 2012;12:278.
[4] Konthur Z, Hust M, Dübel S. Perspectives for systematic in vitro antibody generation. Gene. 2005;364:19–29.
[5] Konthur Z, Wilde J, Lim TS. Semi-automated magnetic bead-based antibody selection from phage display libraries. In: Kontermann R, Dübel S, editors. Antibody engineering. Berlin, Heidelberg: Springer; 2010. p. 267–287.
[6] Ch'ng ACW, Hamidon NHB, Konthur Z, et al. Magnetic nanoparticle-based semi-automated panning for high-throughput antibody selection. In: Hust M, Lim TS, editors. Phage display. Methods in molecular biology. New York (NY): Humana Press; 2018. p. 301–319.
[7] Chin CF, Choong YS, Lim TS. Mass spectrometry immuno assay (MSIA^{TM}) streptavidin disposable automation research tips (DAR T's®) antibody phage display biopanning. In: Hust M, Lim TS, editors. Phage display. Methods in molecular biology. New York (NY): Humana Press, 2018. p. 285–299.
[8] Elgundi Z, Reslan M, Cruz E, et al. The state-of-play and future of antibody therapeutics. Adv Drug Deliv Rev. 2016;122:2–19.
[9] DeFrees S, Wang ZG, Xing R, et al. GlycoPEGylation of recombinant therapeutic proteins produced in *Escherichia coli*. Glycobiology. 2006;16:833–843.
[10] Palmberger D, Rendić D, Tauber P, et al. Insect cells for antibody production: evaluation of an efficient alternative. J Biotechnol. 2011;153:160–166.
[11] Sommaruga S, Lombardi A, Salvade A, et al. Highly efficient production of anti-HER2 scFv antibody variant for targeting breast cancer cells. Appl Microbiol Biotechnol. 2011;91:613–621.
[12] Yao J, Weng Y, Dickey A, et al. Plants as factories for human pharmaceuticals: applications and challenges. IJMS. 2015;16:28549–28565.
[13] Yin G, Garces ED, Yang J, et al. Aglycosylated antibodies and antibody fragments produced in a scalable in vitro transcription-translation system. MAbs. 2012;4:217–225.
[14] Stuart K, Brun R, Croft S, et al. Kinetoplastids: related protozoan pathogens, different diseases. J Clin Invest. 2008;118:1301–1310.
[15] Teixeira S. Control of gene expression in Trypanosomatidae. Braz J Med Biol Res. 1998;31: 1503–1516.
[16] Bates PA. Transmission of Leishmania metacyclic promastigotes by phlebotomine sand flies. Int J Parasitol. 2007;37:1097–1106.
[17] Akhoundi M, Kuhls K, Cannet A, et al. A historical overview of the classification, evolution, and dispersion of Leishmania parasites and sandflies. PLoS Negl Trop Dis. 2016;10:e0004349.

[18] Wallbanks K, Maazoun R, Canning E, et al. The identity of *Leishmania tarentolae* Wenyon 1921. Parasitology. 1985;90:67–78.
[19] Elwasila M. *Leishmania tarentolae* Wenyon, 1921 from the gecko Tarentola annularis in the Sudan. Parasitol Res. 1988;74:591–592.
[20] Novo SP, Leles D, Bianucci R, et al. *Leishmania tarentolae* molecular signatures in a 300 hundred-years-old human Brazilian mummy. Parasit Vectors. 2015;8:1.
[21] Raymond F, Boisvert S, Roy G, et al. Genome sequencing of the lizard parasite *Leishmania tarentolae* reveals loss of genes associated to the intracellular stage of human pathogenic species. Nucleic Acids Res. 2012;40:1131–1147.
[22] Breitling R, Klingner S, Callewaert N, et al. Non-pathogenic trypanosomatid protozoa as a platform for protein research and production. Protein Expr Purif. 2002;25:209–218.
[23] Kushnir S, Gase K, Breitling R, et al. Development of an inducible protein expression system based on the protozoan host *Leishmania tarentolae*. Protein Expr Purif. 2005;42:37–46.
[24] Kovtun O, Mureev S, Jung W, et al. Leishmania cell-free protein expression system. Methods. 2011;55:58–64.
[25] Wiese M, Ilg T, Lottspeich F, et al. Ser/Thr-rich repetitive motifs as targets for phosphoglycan modifications in *Leishmania mexicana* secreted acid phosphatase. EMBO J. 1995;14:1067–1074.
[26] Jones JD. *Leishmania tarentolae*: an alternative approach to the production of monoclonal antibodies to treat emerging viral infections. Infect Dis Poverty. 2015;4:8.
[27] Blackburn EH. Structure and function of telomeres. Nature. 1991;350:569.
[28] Krakow JL, Hereld D, Bangs JD, et al. Identification of a glycolipid precursor of the *Trypanosoma brucei* variant surface glycoprotein. J Biol Chem. 1986;261:12147–12153.
[29] Simpson L, Thiemann OH, Savill NJ, et al. Evolution of RNA editing in trypanosome mitochondria. Proc Natl Acad Sci U S A. 2000;97:6986–6993.
[30] Simpson L, Douglass SM, Lake JA, et al. Comparison of the mitochondrial genomes and steady state transcriptomes of two strains of the trypanosomatid parasite, *Leishmania tarentolae*. PLoS Negl Trop Dis. 2015;9:e0003841.
[31] Klatt S, Konthur Z. Secretory signal peptide modification for optimized antibody-fragment expression-secretion in *Leishmania tarentolae*. Microb Cell Fact. 2012;11:97.
[32] Wong CH. Protein glycosylation: new challenges and opportunities. J Org Chem. 2005;70:4219–4225.
[33] Walsh G. Biopharmaceutical benchmarks 2010. Nat Biotechnol. 2010;28:917.
[34] Roth J, Zuber C, Park S, et al. Protein N-glycosylation, protein folding, and protein quality control. Mol Cells. 2010;30:497–506.
[35] Ferguson MA. The structure, biosynthesis and functions of glycosylphosphatidylinositol anchors, and the contributions of trypanosome research. J Cell Sci. 1999;112:2799–2809.
[36] Khan AH, Bayat H, Rajabibazl M, et al. Humanizing glycosylation pathways in eukaryotic expression systems. World J Microbiol Biotechnol. 2017;33:4.
[37] Ecker DM, Jones SD, Levine HL. The therapeutic monoclonal antibody market. mAbs. 2015;7:9–14.
[38] Jefferis R. Recombinant antibody therapeutics: the impact of glycosylation on mechanisms of action. Trends Pharmacol Sci. 2009;30:356–362.
[39] Reichert JM. Marketed therapeutic antibodies compendium. MAbs. 2012;4:413–415.
[40] Jefferis R. Recombinant proteins and monoclonal antibodies. In: Advances in biochemical engineering/biotechnology. Berlin, Heidelberg: Springer; 2017.
[41] Edelman GM, Cunningham BA, Gall WE, et al. The covalent structure of an entire gammaG immunoglobulin molecule. Proc Natl Acad Sci U S A. 1969;63:78–85.
[42] Alzari P, Lascombe M, Poljak R. Three-dimensional structure of antibodies. Annu Rev Immunol. 1988;6:555–580.
[43] Narciso JET, Uy IDC, Cabang AB, et al. Analysis of the antibody structure based on high-resolution crystallographic studies. N Biotechnol. 2011;28:435–447.
[44] Hanson QM, Barb AW. A perspective on the structure and receptor binding properties of immunoglobulin G Fc. Biochemistry. 2015;54:2931–2942.
[45] Wang Y, Tian Z, Thirumalai D, et al. Neonatal Fc receptor (FcRn): a novel target for therapeutic antibodies and antibody engineering. J Drug Target. 2014;22:269–278.
[46] Nimmerjahn F, Ravetch JV. Fcγ Rs in health and disease. Curr Top Microbiol Immunol. 2011;350:105–125.
[47] Hayes JM, Cosgrave EF, Struwe WB, et al. Glycosylation and Fc receptors. In: Daeron M, Nimmerjahn F, editors. Fc receptors. Current topics in microbiology and immunology. Cham: Springer; 2014. p. 165–199.
[48] Bruhns P, Jönsson F. Mouse and human FcR effector functions. Immunol Rev. 2015;268:25–51.
[49] Jefferis R. Isotype and glycoform selection for antibody therapeutics. Arch Biochem Biophys. 2012;526:159–166.
[50] Subedi GP, Barb AW. The immunoglobulin G1 N-glycan composition affects binding to each low affinity Fcγ receptor. MAbs. 2016;8:1512–1524.
[51] Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. Front Immunol. 2014;5:520.
[52] Reusch D, Tejada ML. Fc glycans of therapeutic antibodies as critical quality attributes. Glycobiology. 2015;25:1325–1334.
[53] Zheng K, Bantog C, Bayer R. The impact of glycosylation on monoclonal antibody conformation and stability. MAbs. 2011;3:568–576.
[54] Liu H, May K. Disulfide bond structures of IgG molecules: structural variations, chemical modifications and possible impacts to stability and biological function. MAbs. 2012;4:17–23.
[55] Wörn A, der Maur AA, Escher D, et al. Correlation between in vitro stability and in vivo performance of

anti-GCN4 intrabodies as cytoplasmic inhibitors. J Biol Chem. 2000;275:2795–2803.
[56] Arbabi-Ghahroudi M, Tanha J, MacKenzie R. Prokaryotic expression of antibodies. Cancer Metastasis Rev. 2005;24:501–519.
[57] Aebi M. N-linked protein glycosylation in the ER. Biochim Biophys Acta. 2013;1833:2430–2437.
[58] Breitling J, Aebi M. N-linked protein glycosylation in the endoplasmic reticulum. Cold Spring Harb Perspect Biol. 2013;5:a013359.
[59] Costa AR, Rodrigues ME, Henriques M, et al. Glycosylation: impact, control and improvement during therapeutic protein production. Crit Rev Biotechnol. 2014;34:281–299.
[60] Higel F, Seidl A, Sörgel F, et al. N-glycosylation heterogeneity and the influence on structure, function and pharmacokinetics of monoclonal antibodies and Fc fusion proteins. Eur J Pharm Biopharm. 2016;100: 94–100.
[61] Spiro RG. Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. Glycobiology. 2002;12:43R–56R.
[62] Liu L. Antibody glycosylation and its impact on the pharmacokinetics and pharmacodynamics of monoclonal antibodies and Fc-fusion proteins. J Pharm Sci. 2015;104:1866–1884.
[63] Zielinska DF, Gnad F, Wiśniewski JR, et al. Precision mapping of an in vivo N-glycoproteome reveals rigid topological and sequence constraints. Cell. 2010;141: 897–907.
[64] Krapp S, Mimura Y, Jefferis R, et al. Structural analysis of human IgG-Fc glycoforms reveals a correlation between glycosylation and structural integrity. J Mol Biol. 2003;325:979–989.
[65] Meier S, Duus J. Carbohydrate dynamics: antibody glycans wiggle and jiggle. Nat Chem Biol. 2011;7: 131–132.
[66] Dalziel M, Crispin M, Scanlan CN, et al. Emerging principles for the therapeutic exploitation of glycosylation. Science. 2014;343:1235681.
[67] Shibata-Koyama M, Iida S, Misaka H, et al. Nonfucosylated rituximab potentiates human neutrophil phagocytosis through its high binding for FcgammaRIIIb and MHC class II expression on the phagocytotic neutrophils. Exp Hematol. 2009;37:309–321.
[68] Lin CW, Tsai MH, Li ST, et al. A common glycan structure on immunoglobulin G for enhancement of effector functions. Proc Natl Acad Sci U S A. 2015; 112:10611–10616.
[69] Borrok MJ, Jung ST, Kang TH, et al. Revisiting the role of glycosylation in the structure of human IgG Fc. ACS Chem Biol. 2012;7:1596–1602.
[70] Lifely MR, Hale C, Boyce S, et al. Glycosylation and biological activity of CAMPATH-1H expressed in different cell lines and grown under different culture conditions. Glycobiology. 1995;5:813–822.
[71] Li H, d'Anjou M. Pharmacological significance of glycosylation in therapeutic proteins. Curr Opin Biotechnol. 2009;20:678–684.
[72] Padler-Karavani V, Yu H, Cao H, et al. Diversity in specificity, abundance, and composition of anti-Neu5Gc antibodies in normal humans: potential implications for disease. Glycobiology. 2008;18: 818–830.
[73] Macher BA, Galili U. The Galα1,3Galβ1,4GlcNAc-R (α-Gal) epitope: a carbohydrate of unique evolution and clinical relevance. Biochim Biophys Acta. 2008; 1780:75–88.
[74] Chung CH, Mirakhur B, Chan E, et al. Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha-1,3-galactose. N Engl J Med. 2008;358: 1109–1117.
[75] Qian J, Liu T, Yang L, et al. Structural characterization of N-linked oligosaccharides on monoclonal antibody cetuximab by the combination of orthogonal matrix-assisted laser desorption/ionization hybrid quadrupole-quadrupole time-of-flight tandem mass spectrometry and sequential enzymatic digestion. Anal Biochem. 2007;364:8–18.
[76] Goh JB, Ng SK. Impact of host cell line choice on glycan profile. Crit Rev Biotechnol. 2017;38(6):1–17.
[77] Basile G, Peticca M. Recombinant protein expression in *Leishmania tarentolae*. Mol Biotechnol. 2009;43: 273–278.
[78] Terpe K. Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. Appl Microbiol Biotechnol. 2006;72:211.
[79] Sørensen HP, Mortensen KK. Soluble expression of recombinant proteins in the cytoplasm of *Escherichia coli*. Microb Cell Fact. 2005;4:1.
[80] Naegeli A, Neupert C, Fan YY, et al. Molecular analysis of an alternative N-glycosylation machinery by functional transfer from *Actinobacillus pleuropneumoniae* to *Escherichia coli*. J Biol Chem. 2014;289: 2170–2179.
[81] Wacker M, Linton D, Hitchen PG, et al. N-linked glycosylation in *Campylobacter jejuni* and its functional transfer into *E. coli*. Science. 2002;298:1790–1793.
[82] Ghaderi D, Zhang M, Hurtado-Ziola N, et al. Production platforms for biotherapeutic glycoproteins. Occurrence, impact, and challenges of non-human sialylation. Biotechnol Genet. 2012;28:147–176.
[83] Vaughn J, Goodwin R, Tompkins G, et al. The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera; Noctuidae). In Vitro. 1977;13: 213–217.
[84] Wickham T, Davis T, Granados R, et al. Screening of insect cell lines for the production of recombinant proteins and infectious virus in the baculovirus expression system. Biotechnol Prog. 1992;8:391–396.
[85] Davis TR, Wickham TJ, McKenna KA, et al. Comparative recombinant protein production of eight insect cell lines. In Vitro Cell Dev Biol Anim. 1993;29a:388–390.
[86] Pennock G, Shoemaker C, Miller L. Strong and regulated expression of *Escherichia coli* beta-galactosidase in insect cells with a baculovirus vector. Mol Cell Biol. 1984;4:399–406.
[87] Luckow VA, Lee S, Barry G, et al. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. J Virol. 1993;67:4566–4579.

[88] Cérutti M, Golay J. Lepidopteran cells, an alternative for the production of recombinant antibodies? MAbs. 2012;4:294–309.
[89] Hsu TA, Takahashi N, Tsukamoto Y, et al. Differential N-glycan patterns of secreted and intracellular IgG produced in Trichoplusia ni cells. J Biol Chem. 1997; 272:9062–9070.
[90] Geisler C, Aumiller JJ, Jarvis DL. A fused lobes gene encodes the processing beta-N-acetylglucosaminidase in Sf9 cells. J Biol Chem. 2008;283:11330–11339.
[91] Kim YK, Kim KR, Kang DG, et al. Expression of β-1,4-galactosyltransferase and suppression of β-N-acetylglucosaminidase to aid synthesis of complex N-glycans in insect Drosophila S2 cells. J Biotechnol. 2011; 153:145–152.
[92] Hollister JR, Jarvis DL. Engineering lepidopteran insect cells for sialoglycoprotein production by genetic transformation with mammalian β1, 4-galactosyltransferase and α2, 6-sialyltransferase genes. Glycobiology. 2001;11:1–9.
[93] Mattanovich D, Branduardi P, Dato L, et al. Recombinant protein production in yeasts. In: Lorence A, editor. Recombinant gene expression. Methods in molecular biology. Totowa (NJ): Humana Press; 2011. p. 329–358.
[94] Wildt S, Gerngross TU. The humanization of N-glycosylation pathways in yeast. Nat Rev Microbiol. 2005; 3:119.
[95] Chiba Y, Suzuki M, Yoshida S, et al. Production of human compatible high mannose-type (Man5GlcNAc2) sugar chains in *Saccharomyces cerevisiae*. J Biol Chem. 1998;273:26298–26304.
[96] Hamilton SR, Gerngross TU. Glycosylation engineering in yeast: the advent of fully humanized yeast. Curr Opin Biotechnol. 2007;18:387–392.
[97] Hamilton SR, Bobrowicz P, Bobrowicz B, et al. Production of complex human glycoproteins in yeast. Science. 2003;301:1244–1246.
[98] Gerngross TU. Advances in the production of human therapeutic proteins in yeasts and filamentous fungi. Nat Biotechnol. 2004;22:1409–1414.
[99] Li H, Sethuraman N, Stadheim TA, et al. Optimization of humanized IgGs in glycoengineered *Pichia pastoris*. Nat Biotechnol. 2006;24:210.
[100] Jin C, Altmann F, Strasser R, et al. A plant-derived human monoclonal antibody induces an anti-carbohydrate immune response in rabbits. Glycobiology. 2007;18:235–241.
[101] Cox KM, Sterling JD, Regan JT, et al. Glycan optimization of a human monoclonal antibody in the aquatic plant *Lemna minor*. Nat Biotechnol. 2006;24:1591.
[102] Castilho A, Strasser R, Stadlmann J, et al. In planta protein sialylation through overexpression of the respective mammalian pathway. J Biol Chem. 2010; 285:15923–15930.
[103] Chen Q. Glycoengineering of plants yields glycoproteins with polysialylation and other defined N-glycoforms. Proc Natl Acad Sci U S A. 2016;113:9404–9406.
[104] Zeitlin L, Pettitt J, Scully C, et al. Enhanced potency of a fucose-free monoclonal antibody being developed as an Ebola virus immunoprotectant. Proc Natl Acad Sci U S A. 2011;108:20690–20694.
[105] Wang Q, Chung CY, Chough S, et al. Antibody glycoengineering strategies in mammalian cells. Biotechnol Bioeng. 2018;115:1378–1393.
[106] Ezure T, Suzuki T, Shikata M, et al. A cell-free protein synthesis system from insect cells. In: Endo Y, Takai K, Ueda T, editors. Cell-free protein production. Methods in molecular biology. Humana Press; 2010. p. 31–42.
[107] Sissons CH. Yeast protein synthesis. Preparation and analysis of a highly active cell-free system. Biochem J. 1974;144:131–140.
[108] Arduengo M, Schenborn E, Hurst R. The role of cell-free rabbit reticulocyte expression systems in functional proteomics. Cell-free expression. Austin (TX): Landes Bioscience; 2007. p. 1–18.
[109] Brodel AK, Sonnabend A, Kubick S. Cell-free protein expression based on extracts from CHO cells. Biotechnol Bioeng. 2014;111:25–36.
[110] Johnston WA, Alexandrov K. Production of eukaryotic cell-free lysate from *Leishmania tarentolae*. In: Alexandrov K, Johnston WA, editors. Cell-free protein synthesis. Methods in molecular biology. Totowa (NJ): Humana Press; 2014. p. 1–15.
[111] Hunter DJB, Bhumkar A, Giles N, et al. Unexpected instabilities explain batch-to-batch variability in cell-free protein expression systems. Biotechnol Bioeng. 2018;115:1904–1914.
[112] Jørgensen ML, Friis NA, Just J, et al. Expression of single-chain variable fragments fused with the Fc-region of rabbit IgG in *Leishmania tarentolae*. Microb Cell Fact. 2014;13:9.
[113] Lee MGS, Van der Ploeg LH. Transcription of protein-coding genes in trypanosomes by RNA polymerase I. Annu Rev Microbiol. 1997;51:463–489.
[114] Wirtz E, Hartmann C, Clayton C. Gene expression mediated by bacteriophage T3 and 17 RNA polymerases in transgenic trypanosomes. Nucl Acids Res. 1994;22:3887–3894.
[115] Misslitz A, Mottram JC, Overath P, et al. Targeted integration into a rRNA locus results in uniform and high level expression of transgenes in *Leishmania amastigotes*. Mol Biochem Parasitol. 2000;107: 251–261.
[116] Kushnir S, Cirstea IC, Basiliya L, et al. Artificial linear episome-based protein expression system for protozoon *Leishmania tarentolae*. Mol Biochem Parasitol. 2011;176:69–79.
[117] Beverley SM, Clayton CE. Transfection of Leishmania and *Trypanosoma brucei* by electroporation. In: Hyde JE, editor. Protocol in molecular parasitology. Methods in molecular biology. Humana Press; 1993. p. 333–348.
[118] Gazdag EM, Cirstea IC, Breitling R, et al. Purification and crystallization of human Cu/Zn superoxide dismutase recombinantly produced in the protozoan *Leishmania tarentolae*. Acta Crystallogr F Struct Biol Cryst Commun. 2010;66:871–877.
[119] Phan HP, Sugino M, Niimi T. The production of recombinant human laminin-332 in a *Leishmania tarentolae* expression system. Protein Expr Purif. 2009; 68:79–84.

[120] Klatt S, Rohe M, Alagesan K, et al. Production of glycosylated soluble amyloid precursor protein alpha (sAPPalpha) in *Leishmania tarentolae*. J Proteome Res. 2013;12:396–403.

[121] Just J, Lykkemark S, Nielsen CH, et al. Pericyte modulation by a functional antibody obtained by a novel single-cell selection strategy. Microcirculation. 2017; 24:e12365.

[122] Pereira M. A developmentally regulated neuraminidase activity in *Trypanosoma cruzi*. Science. 1983;219: 1444–1446.

[123] Schenkman S, Eichinger D, Pereira M, et al. Structural and functional properties of Trypanosoma transsialidase. Annu Rev Microbiol. 1994;48:499–523.

[124] Grilo AL, Mantalaris A. The increasingly human and profitable monoclonal antibody market. Trends Biotechnol. 2018;37:9–16.