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Optimization of erythropoietin production with controlled glycosylation-PEGylated erythropoietin produced in glycoengineered *Pichia pastoris*

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ABSTRACT

Pichia pastoris is a methylotropic yeast that has gained great importance as an organism for protein expression in recent years. Here, we report the expression of recombinant human erythropoietin (rhEPO) in glycoengineered *P. pastoris*. We show that glycosylation fidelity is maintained in fermentation volumes spanning six orders of magnitude and that the protein can be purified to high homogeneity. In order to increase the half-life of rhEPO, the purified protein was coupled to polyethylene glycol (PEG) and then compared to the currently marketed erythropoiesis stimulating agent, Aranesp® (darbepoetin). In *in vitro* cell proliferation assays the PEGylated protein was slightly, and the non-PEGylated protein was significantly more active than comparator. Pharmacodynamics as well as pharmacokinetic activity of PEGylated rhEPO in animals was comparable to that of Aranesp®. Taken together, our results show that glycoengineered *P. pastoris* is a suitable production host for rhEPO, yielding an active biologic that is comparable to those produced in current mammalian host systems.

1. Introduction

Erythropoietin (EPO) is a 30.4 kDa glycoprotein hormone (Davis et al., 1987) with pleiotropic functions including differentiation of erythroid progenitor cells (Stephenson et al., 1971; Goldwasser, 1984), inhibition of apoptosis (Koury and Bondurant, 1990), and stimulation of cell proliferation (Seong et al., 2006; Sigounas et al., 1997). It contains three N-linked glycans (N24, N38 and N83) and one *O*-linked glycan (S126) which contribute approximately 40% to its molecular weight (Takeuchi et al., 1988). In humans, erythropoietin is produced by peritubular cells in the adult kidney, and in hepatocytes in the fetus (Fisher, 2003). Upon production, erythropoietin travels via the circulatory system to the bone marrow where it engages its cognate receptor on specific progenitor cells to stimulate erythropoiesis (Fisher, 2003). The market for erythropoietin has seen a steady increase for all indications: oncology and chronic kidney disease, dialysis and pre-dialysis. In a recently published data review, four of the top ten biotech drugs were a form of recombinant erythropoietin, with Aranesp® ranking third overall in global sales (Lawrence, 2006).

Currently marketed forms of recombinant erythropoietin include Epogen with three N-glycan structures and Aranesp® (darbepoetin), an epoetin engineered to contain two additional N-glycosylation sites, conferring greater metabolic stability *in vivo* (MacDougall et al., 1999) (Fig. 1). CHO cell-produced epoetin are secreted with a heterogeneous mixture of sialylated N-glycan structures. Usually the manufacturing process is controlled to enrich for the tetra-antennary sialylated glycoforms, which, along with tri-antennary forms, are required for maximum *in vivo* efficacy (Egrie and Browne, 2001). However, in cell-based and receptor binding assays, tri- and tetra-sialylated erythropoietin and

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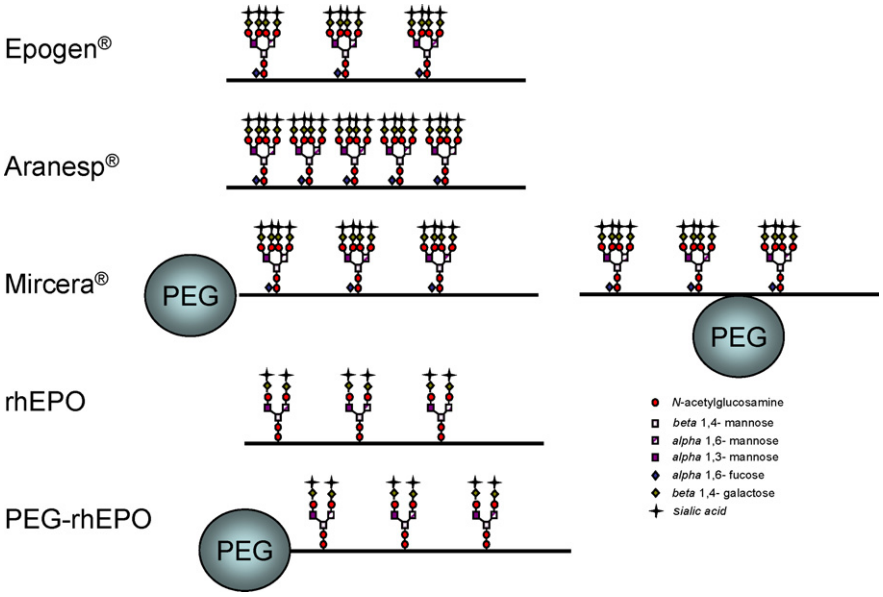


Fig. 1. Schematic structure of the three currently marketed erythropoiesis stimulating agents Epogen®, Aranesp®, Mircera®, and the PEGylated and non-PEGylated forms of rhEPO produced in glycoengineered *P. pastoris*.

darbepoetin exhibit decreased potency relative to bi-antennary sialylated erythropoietin (Takeuchi et al., 1989). This paradox can be explained by the extended serum half-lives of the tetra-antennary sialylated erythropoietin and darbepoetin compared with the faster clearance rate of bi-antennary sialylated erythropoietin.

Recently a third-generation erythropoietin, Continuous Erythropoietin Receptor Activator (CERA), was developed by adding a polyethylene glycol (PEG) chain through amide bonds to the N-terminal amino group or the ε-amino group of lysines (predominantly lysine-52 or lysine-45) of the molecule (MacDougall, 2005).

Here we report that GlycoFi has engineered strains of *Pichia pastoris* capable of producing recombinant erythropoietin with terminally sialylated bi-antennary N-glycan structures. Erythropoietin with bi-antennary glycans has enhanced *in vitro* activity when compared to Arasnesp®. However, its potentially higher *in vivo* efficacy cannot be realized because of its faster clearance due to the small size. To translate the enhanced *in vitro* activity of bi-antennary erythropoietin to increased *in vivo* efficacy it is necessary to overcome the limitations of a smaller erythropoietin molecule. We show that this is possible by increasing the mass of sialylated bi-antennary erythropoietin with conjugation of polyethylene glycol to retain the desired pharmacokinetic characteristics.

2. Experimental procedures

2.1. Strain construction

Detailed information on construction of production strains is given in Supplemental File 1.

2.2. Bioreactor cultivations

Cultivations were done in 0.5-l (Sixfors®, ATR Biotech, Laurel, MD), 3-l (Applikon, Foster City, CA) and 15-l (Applikon, Foster City, CA) glass bioreactors as well as a 40-l (Applikon, Foster City, CA), 2000-l and 5000-l stainless steel, steam-in-place bioreactor. Seed cultures were prepared by inoculating BMGY media (4% glycerol, 1% yeast extract (BD, Franklin Lakes, NJ), 2% peptone (BD, Franklin Lakes, NJ), 1.19% potassium phosphate monobasic (Fisher, Fairlawn, NJ), 0.23% potassium phosphate dibasic (EMD Chemical, Inc, Gibbstown, NJ), 1.82% D-sorbitol (Acros Organics, NJ) 1.34% yeast nitrogen base (BD, Franklin Lakes, NJ) and 4×10^{-5}% D-biotin, pH adjusted to 6.5) directly with frozen stock vials at a 0.1% volumetric ratio. Seed flasks were incubated at 24 °C for 48 h at 180 RPM to obtain an optical density (OD600) of 20 ± 5 to ensure that cells were growing exponentially upon transfer. The bioreactor was inoculated with a 10% volumetric ratio of seed to initial media (BMGY). Cultivations were done in fed-batch mode under the following conditions: temperature set at 24 ± 0.5 °C, pH controlled at 6.5 ± 0.1 with 30% NH_4OH, dissolved oxygen was maintained at 1.7 ± 0.1 mg l^{-1} by cascading agitation rate on the addition of pure O_2 to the fixed airflow rate of 0.7 vvm. After depletion of the initial charge glycerol (40 g l^{-1}), a 60 g l^{-1} of 50% (w/w) glycerol solution containing 12.5 ml l^{-1} of PTM1 salts (65 g $FeSO_4 \cdot 7H_2O$, 20 g $ZnCl_2$, 9 g H_2SO_4, 6 g $CuSO_4 \cdot 5H_2O$, 5 g H_2SO_4, 3 g $MnSO_4 \cdot 7H_2O$, 500 mg $CoCl_2 \cdot 6H_2O$, 200 mg $NaMoO_4 \cdot 2H_2O$, 200 mg biotin, 80 mg NaI, 20 mg H_3BO_4 l^{-1}) and 12.5 ml l^{-1} of D-biotin (400 mg l^{-1} stock solution) was added into the reactor as a bolus shot. Glycerol was consumed within 3–4 h of the bolus addition which was indicated by a DO (dissolved oxygen) spike. At the end of the glycerol bolus phase, wet cell weight (WCW) reached around 180 ± 20 g l^{-1}. At the end of the batch phase, start of induction, and 24 h into induction, 1.33 ml l^{-1} of methanol stock solution containing 1.95 mg/ml PMTi-3 (a synthetic inhibitor of fungal *O*-glycosylation) and 2.5 mg/ml Pepstatin A (protease inhibitor) was added into the reactor. Induction was initiated after a 30 min starvation phase when methanol (containing 12.5 ml l^{-1} of PTM1 salts and 12.5 ml l^{-1} of D-biotin (400 mg l^{-1} stock solution)) was fed at a flat rate of 2.34 g l^{-1} h^{-1} until the end of the 40 h induction period (WCW reached around 260 ± 20 g l^{-1}). The reactor was harvested by centrifugation (15 810 × *g*, for 30 min), before the target protein was captured using the first step purification (Potgieter et al., 2009).

2.3. Protein purification and PEGylation

PEGylated recombinant human EPO (PEG-rhEPO) was generated by a multi-step chromatographic separation. After clarification by centrifugation in combination with 0.45 μm microfiltration to remove the cell mass, the filtrate was further processed and purified according to the scheme in Supplemental Fig. 3 in dependence

Table 1
Glycosylation differences between rhEPO produced in CHO and glycoengineered *P. pastoris*.

	CHO	Glycoengineered *P. pastoris*	Human
Antennae	2–4	2	2–3 (mainly 2)
Polylactosamine	Yes (heterogeneous)	No	No
Sialic acid linkage	α 2–3[a]	α 2–6[a]	α 2–3 and α 2–6[a]
Core fucose	Yes (heterogeneous)	No	Yes
NGNA (non-human)	Yes	No	No
O-glycosylation	*O*-GalNAc[b] (heterogeneous)	*O*-mannose (below detection limit of HPAEC-PAD)	*O*-GalNac, partially occupied

[a] Human contains both α 2–3 and α 2–6, predominant is α 2–6 linkage.
[b] A substantial fraction of human serum EPO lacks *O*-glycosylation at Ser-126 while the rhEPO produced in CHO is fully occupied (Skibeli et al., 2001).

of the required purity and processing scale (lab vs. pilot production scale).

The N-terminus of the purified hEPO molecule was preferentially conjugated to a 40-kDa linear polyethylene glycol (PEG) via reductive amination (PEGylation) (Kinstler et al., 1996). A solution of rhEPO at 1 mg/ml in 20 mM sodium acetate pH 5.0 was added to a vial containing 40 kDa methoxy polyethylene glycol propionaldehyde (10 mg/ml). Once the PEG was dissolved, sodium cyanoborohydride (20 mM) was added to the reaction mixture. The reaction was stirred at 4 °C for 16 h and then diluted to 1:10 with 1 mM HCl (final protein concentration of 0.1 mg/ml) and loaded onto a SP sepharose FF, cation exchange column pre-equilibrated with 20 mM sodium acetate, pH 5.0 buffer. The column was washed with three column volumes of 20 mM sodium acetate pH 5.0 buffer before a linear gradient to 50% with 20 mM sodium acetate pH 5.0 + 1 M NaCl buffer was applied over 25 column volumes. The fractions having an absorbance at 280 nm when analyzed by reducing SDS–PAGE showed a clear separation of mono-PEGylated from un-PEGylated and multi-PEGylated forms of rhEPO. The fractions containing mono-PEGylated rhEPO were collected and diafiltered into the final formulation buffer (20 mM sodium phosphate, 120 mM sodium chloride, 0.005% polysorbate 20 (w/v), pH 7.0). Protein quantitation for purified rhEPO as well as PEGylated rhEPO was carried out by UV spectroscopy at 280 nm using the absorbency value of 1.23 l g^{-1} cm^{-1} as the extinction coefficient for a 1 mg/ml solution. The mono-PEGylated rhEPO (final product) was diluted to a pre-determined concentration and sterile filtered into the drug substance storage container. The drug substance was stored at 2–8 °C until filling, at which time it was aseptically filled into glass vials that were then sealed with a rubber stopper and aluminum cap and stored at 2–8 °C until use.

2.4. Analytical characterization

Recombinant hEPO protein was analyzed by SDS–PAGE according to Laemmli (1970) and stained for visualization with Coomassie blue. For glycan analysis, the glycans were released from rhEPO by treatment with PNGase F. Released glycans were labeled with 2-aminobenzamide (2-AB) and analyzed by HPLC following the conditions previously described by Li et al. (2007) with the following modifications. HPLC analysis of the labeled glycans was performed on an Agilent 1200 using an Alltech Prevail Carbohydrate ES 5 μm 250 mm × 4.6 mm column with a flow rate of 1.3 ml/min at 45 °C. The gradient was extended to 75 min to elute both neutral, mono- and bi-sialylated glycans. rhEPO protein intact mass was confirmed using a Thermo Electron Finnigan LTQ mass spectrometer equipped with a Thermo Electron Finnigan Surveyor HPLC system by running the deglycosylated EPO on an Agilent Poroshell 300SB C8.

The intactness and presence of aggregation of the purified rhEPO was determined by size exclusion high-performance liquid chromatography (SEC–HPLC) using a Thermo Finnigan Surveyor system and a GE Healthcare Superdex 200 10/300 GL column. The HPLC gradient consisted of a 30 min isocratic run at room temperature with a flow rate of 0.45 ml/min using a buffer of 100 mM sodium phosphate (pH 6.8), 150 mM NaCl, and 0.05% sodium azide.

2.5. Erythropoietin mediated proliferation in TF-1 cells

To assess the ability of *P. pastoris* rhEPO to support the proliferation of erythropoietin-sensitive TF-1 cells, a multi-well plate cell based assay was employed which provides a fluorometric method for estimating the number of viable cells utilizing the CellTiter-Blue™ reagent (Promega). Briefly, cells were washed once in PBS and cultured overnight in Optimem media with 0.5% FCS at a concentration of $5e^5$ cells/ml. The cells were harvested and seeded at $1\ e^3$/190 μl in black 96-well microtiter plates. Next, 10 μl of recombinant erythropoietins, at 20× the final desired concentration, were added to the cell suspension, mixed, and incubated with the cells for up to 7 days at 37 °C. Then, plates were spun down and 100 μl of culture supernatant was removed and 20 μl of CellTiter-Blue reagent was added to each well. Plates were briefly shaken and then incubated for 2 h prior to the fluorescence being read at an excitation and emission of 550 and 600 nm, respectively. Data were analyzed by GraphPad Prism.

2.6. Human erythroid colony formation and proliferation assay

Purified human CD34+ bone marrow cells (lot #051063 purchased from Poietics) were stored in a N_2 gaseous freezer. Cells were thawed rapidly at 37 °C on the day of the experiment and the contents of the vial diluted in 10 ml of Iscove's medium containing 2% FBS and washed by centrifugation (1200 rpm for 10 min, room temperature). The supernatant was discarded and the cell pellet resuspended in a known volume of Iscove's medium containing 2% FBS. A cell count (3% glacial acetic acid) and viability assessment (trypan blue) were performed. Clonogenic progenitors of the erythroid (CFU-E and BFU-E) and myeloid (CFU-GM) lineages were set up in methylcellulose-based culture media, MethoCult™ 4230NS, containing sub-optimal concentration of myeloid cytokines (SCF at 5 ng/ml and IL-3 at 1 ng/ml). All test EPOs were added to obtain final concentrations of 30, 3, 0.3, 0.1, 0.03 and 0.01 ng/ml. A positive control containing saturating concentrations of erythroid and myeloid cytokines, MethoCult™ 84434, and a negative control (MethoCult™ 4230NS) containing no added EPO were also initiated. All conditions were set up in triplicate with 800 CD34+ human bone marrow cells per culture and incubated at 37 °C, 5% CO_2. On day 14, colony numbers were scored and assessed microscopically by trained personnel.

2.7. Pharmacodynamics in normal mice

All animal studies were performed according to the Institutional Animal Care and Use Committee guidelines. Baseline hemoglobin levels were determined in female CD-1 mice (Charles River) using an Advia 120. One week later, PEG-rhEPO or Aranesp® was delivered via intraperitoneal (IP) injection to mice at various doses (molar equivalent doses of rhEPO and Aranesp® based on protein

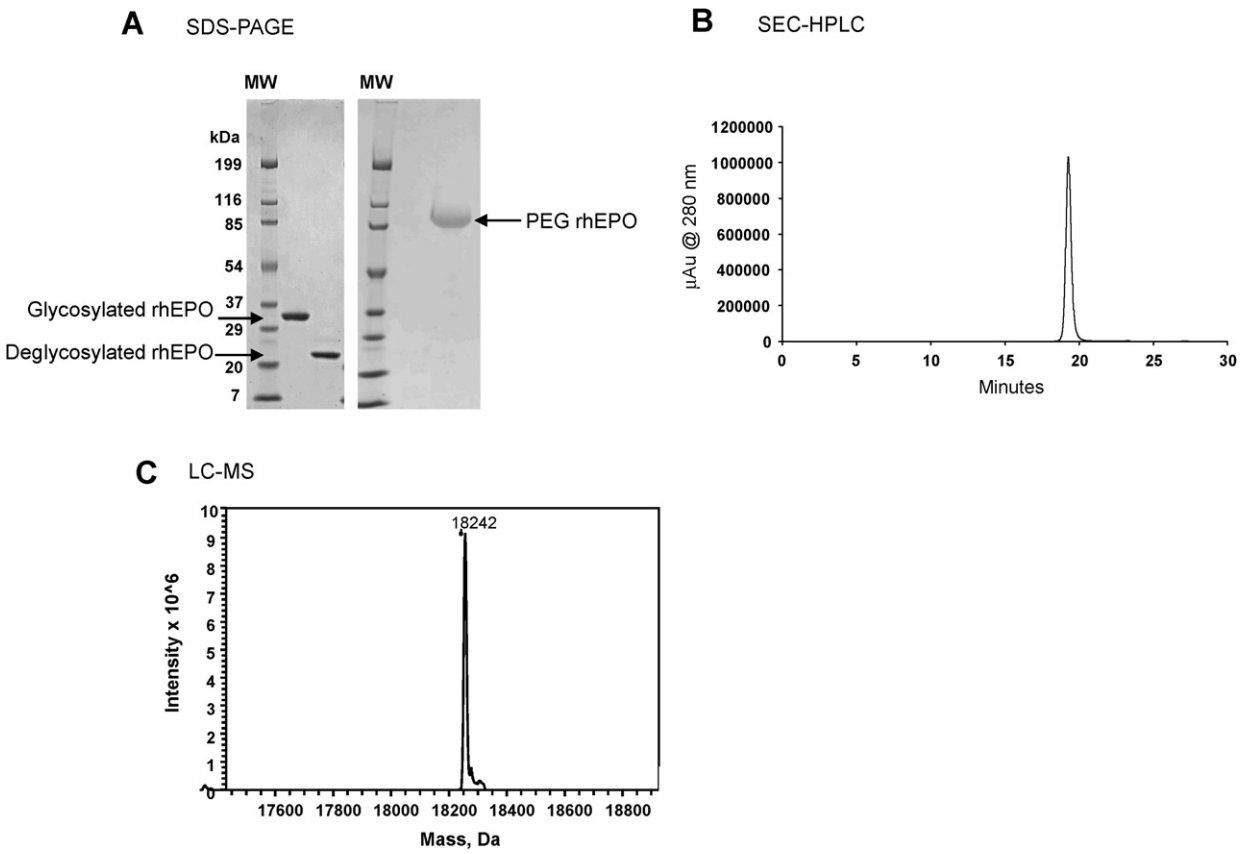


Fig. 2. (A) SDS–PAGE of glycosylated rhEPO, deglycosylated rhEPO and PEG-rhEPO. MW: molecular weight marker. (B) SEC–HPLC of rhEPO. (C) LC–MS determination of molecular weight of deglycosylated rhEPO.

mass). After seven days animals were euthanized and hemoglobin levels were measured (using an ADVIA 120 hematology analyzer). The studies were vehicle controlled (100 μg/ml HSA in 0.9% NaCl), with dilutions of Aranesp® or rhEPO being made in the same vehicle.

2.8. Pharmacokinetics in rats

Pharmacokinetic studies in rats: Sprague-Dawley rats (4 males and 4 females per group) were dosed with PEG-rhEPO at 3 μg/kg or Aranesp® at 4.5 μg/kg through IV or SC injection. Serum samples were obtained at 0.5, 1, 2, 4, 8, 24, 48 and 96 h post dose. The serum rhEPO concentrations were determined with Quantikine IVD human erythropoietin kits (R&D systems).

3. Results

3.1. Construction of a P. pastoris production strain expressing rhEPO with bi-antennary sialylated glycans

Construction of the rhEPO production strain was accomplished with a main focus on three areas of strain development: (A) generation of homogeneous bi-antennary sialylated glycans over a large range of expression scales, (B) maximization of recoverable rhEPO expression, and (C) elimination of potentially immunogenic glycan structures.

(A) Using previously described glycosylation enzyme/leader combinations and screening procedures (Nett et al., 2011) we identified YGLY1703 as a strain with exquisitely homogeneous bi-antennary galactosylated glycans. In order to be able to screen for optimal sialic acid transfer onto those glycans we first transformed the strain with a plasmid encoding rhEPO and then a second plasmid containing the five genes necessary for the production of CMP-sialic acid, its transport into the Golgi apparatus and the transfer of sialic acid onto the galactosylated protein. We then screened 384 transformants for maximum sialic acid transfer (as determined by MALDI TOF) at the 96-well scale (as described in Nett et al., 2011), 21 of them at the 0.5 l fermentation scale, and 5 of those in 3 l fermentations, upon which we identified strain YGLY3159 with highly homogeneous glycans. We have chosen not to engineer the fucosylation pathway into *P. pastoris* since core fucosylation has not been reported to impact the activity of EPO. Alpha 2–6 sialic acid transferase was engineered into *P. pastoris* because the α 2–6-linkage is the predominant sialic acid linkage in humans (Table 1, Varki, 2010). (B) In order to maximize rhEPO expression we added a second expression plasmid with a different signal sequence and selectable marker to the development strain YGLY3159 (for details see Supplemental File 1). qPCR and deep sequencing (results not shown) allowed us to estimate the copy numbers of each set of expression plasmids that was integrated into the strains. While the first expression plasmid using the Zeocin marker added approximately 4–5 copies, the second expression plasmid using the ADE2* marker added another 7–8 copies for a total of 12–13 copies of the rhEPO expression cassette. This essentially doubled the fermentation titer of 25 mg l^{-1} for YGLY3159 to 50 mg l^{-1} for YGLY7117. (C) During characterization of rhEPO expressed using YGLY3159 we discovered an impurity that cross-reacted with antibodies raised against cells without a rhEPO expression cassette (no ORF strain). This cross reactivity disappeared when the sample was pretreated with PNGase F, suggesting that the antigenic epitope had been a glycan. We reasoned that, although during construction of YGLY3159 we had knocked out the gene for *BMT2* (a beta mannosyltransferase), the remaining genes in the family (*BMT1*, *BMT3*, *BMT4*) might be responsible for the addition of the antigenic glycans (Mille

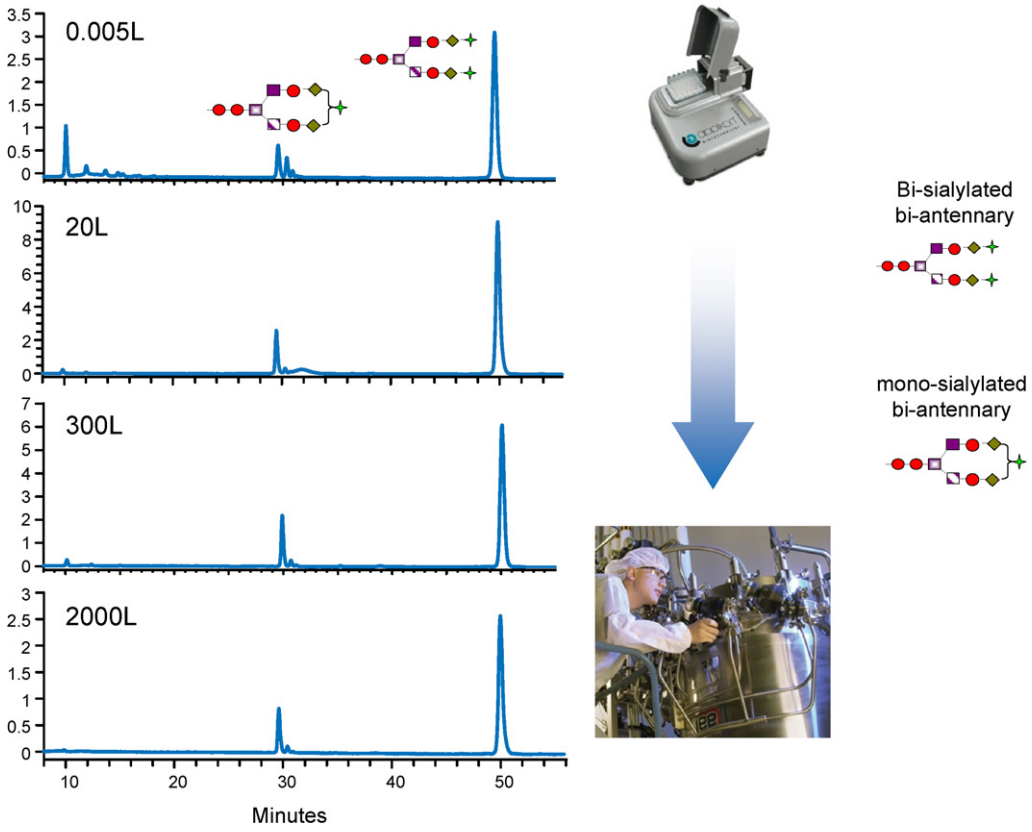


Fig. 3. HPLC analysis of 2-AB-labeled N-glycans released from rhEPO produced at various scales.

et al., 2008). Upon further knockout of these genes, rhEPO isolated from the resulting production strain YGLY7117 did not exhibit any reactivity to the above antibody anymore, confirming our theory that a high mannose beta-glycan had been responsible for the cross-reactivity (Hopkins et al., 2011).

3.2. rhEPO expressed in glycoengineered P. pastoris can be purified and mono-PEGylated to a high degree of purity and homogeneity

rhEPO from fermentation supernatant of strain YGLY7117 was purified according to the scheme detailed in Supplemental Fig. 3. On SDS gels the material appeared as a single band with the expected shift to a lower molecular band upon removal of N-glycans using PNGase F (Fig. 2A). Purity was estimated by SEC–HPLC to be greater than 98%, and intactness was confirmed by LC–MS (see Fig. 2B and C). The purified rhEPO molecule was then conjugated to a 40 kDa linear PEG (as described in Section 2) and the predominantly mono-PEGylated form was isolated using cation exchange chromatography. Peptide mapping and N-terminal sequencing (results not shown) indicated that the PEG had predominantly been conjugated to the N-terminus of the rhEPO protein. On SDS gels the PEGylated protein ran as a diffuse band of significantly higher molecular weight than unconjugated rhEPO (Fig. 2A).

3.3. Glycosylation fidelity of N-glycans is maintained irrespective of fermentation scale

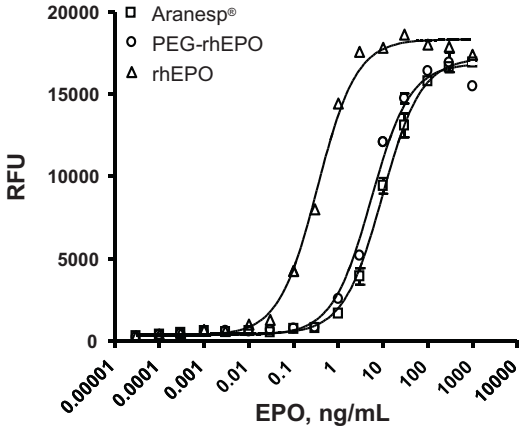
Consistency and reproducibility of protein glycosylation are of utmost importance to the efficacy and manufacturing of rhEPO. It is well established that a change in mammalian cell culture conditions can significantly affect the composition and quality of rhEPO batches (Yuen et al., 2003). To determine the robustness of our glycoengineered *P. pastoris* strains at different scales, we tested them in fermentation vessels spanning six orders of magnitude. Strain YGLY3159 was used for fermentation studies and taken from lab scale to increasingly larger fermenters up to a production scale of 2000 l. Analysis of the glycans released from rhEPO purified by blue dye-affinity and hydroxyapatite chromatography shows that the high level of sialic acid transfer is maintained from 5 ml mini fermenters to the 2000 l production scale (Fig. 3).

3.4. rhEPO produced in glycoengineered P. pastoris is predominantly free of O-glycans

Recombinant human EPO produced in mammalian cell culture has a single mucin-type *O*-linked glycan attached to serine 126. When Stübinger and colleagues examined the *O*-glycan on a commercial preparation of Aranesp® by removal of the N-glycans with PNGase F and analysis of the resulting material, they found that this glycan is heterogeneous in terms of both site occupancy at S126 as well as the extension of Ser-*O*-GalNAc and sialic acid content (Stübinger et al., 2005). rhEPO produced in glycoengineered *P. pastoris* and treated the same way on the other hand, is predominantly free of *O*-glycans as demonstrated by LC–MS analysis (Fig. 2C). The level of *O*-linked mannose is below the level of detection using the HPAEC-PAD method (Table 1, Stadheim et al., 2008).

3.5. In vitro activity and potency of rhEPO produced in glycoengineered P. pastoris

The functional *in vitro* potency of *P. pastoris* rhEPO was assessed in a cell based assay which evaluated downstream signaling of endogenous human EPO-receptor (EPO-R) leading to cellular proliferation in EPO responsive TF-1 cells. In this regard, the *in vitro* activity of *P. pastoris* rhEPO was compared to that of another



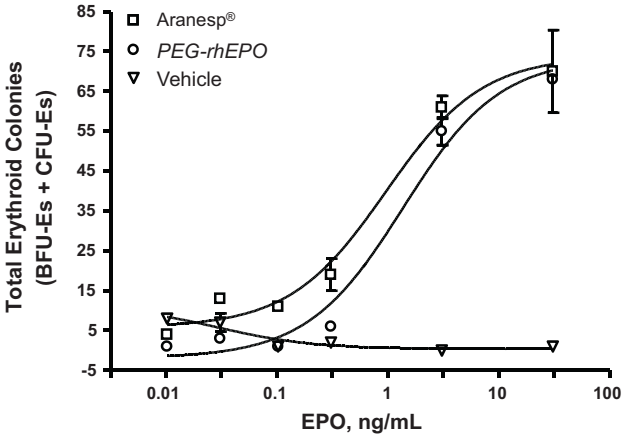
	Aranesp®	PEG-rhEPO	rhEPO
EC50 (ng/mL)	9.63	5.42	0.34

Fig. 4. EPO induced proliferation in TF-1 cells. Increasing concentrations of PEG-rhEPO, rhEPO, or Aranesp® were incubated with TF-1 cells. After 7 days in culture, proliferation was measured utilizing the CellTiter-Blue® reagent. The data represent means of replicate samples.

biologic response modifier and erythropoiesis stimulating agent, Aranesp®. *P. pastoris* PEG-rhEPO was found to be slightly more potent than Aranesp® in its ability to stimulate EPO dependent proliferation (EC_{50s} of 5.4 ng/ml for *P. pastoris* PEG-rhEPO and 9.6 ng/ml for Aranesp®, respectively, Fig. 4). In addition, consistent with previously published data (Takeuchi et al., 1989), bi-antennary EPO (*P. pastoris* rhEPO (no PEG)) showed significantly more potent *in vitro* activity compared to predominantly tri- and tetra-antennary EPO (Aranesp®) (0.34 ng/ml and 9.6 ng/ml, respectively). Not surprisingly, the addition of PEG to bi-antennary EPO also reduced its potency (0.34 ng/ml compared to 5.4 ng/ml). In addition, the functional potency of *P. pastoris* PEG-rhEPO in promoting the proliferation and differentiation of primary lineage-specific human erythroid progenitors from CD34+ precursors was assessed in a hematopoietic colony forming *in vitro* culture system. The bioactivity of *P. pastoris* PEG-rhEPO in this regard was assessed relative to Aranesp®. *P. pastoris* PEG-rhEPO was found to have similar activity to Aranesp® in its ability to promote the proliferation and differentiation of human erythroid progenitor cells (EC_{50s} of 1.34 ng/ml for *P. pastoris* PEG-rhEPO and 0.94 ng/ml for Aranesp®, respectively) from human CD34+ stem cell precursors (Fig. 5).

3.6. PEG-rhEPO produced in glycoengineered P. pastoris has pharmacodynamic activity in mice comparable to that of Aranesp®

The *in vivo* functional potency of PEG-rhEPO was evaluated in a normocythemic mouse model using dose-dependent stimulation of erythropoietic responses (hemoglobin) as an indicator of short term bioactivity. Aranesp® served as a comparator in these assessments. In order to evaluate the *in vivo* bioactivity of PEG-rhEPO mice were injected with *P. pastoris* PEG-rhEPO or Aranesp® via IP route at 0.3, 1.0, 3.0, 10.0 or 30.0 μg/kg (molar equivalent based on protein mass). As shown in Fig. 6, PEG-rhEPO is a potent stimulator of erythropoiesis in normocythemic mice *in vivo*. PEG-rhEPO significantly increases hemoglobin levels at doses above 0.3 μg/kg. In addition, PEG-rhEPO is comparable to Aranesp® in this regard.



	Aranesp®	PEG-rhEPO
EC50 (ng/mL)	0.94	1.34

Fig. 5. Activity in human erythroid colony formation and proliferation assay. Increasing concentrations of PEG-rhEPO and Aranesp® were incubated with human CD34+ cells. On day 14, colony numbers were scored and assessed microscopically by trained personnel. The data represent means of triplicate samples ± SD.

3.7. Pharmacokinetics of PEG-rhEPO produced in glycoengineered P. pastoris

The pharmacokinetics (PK) of PEG-rhEPO was compared to Aranesp® following single dose IV and SC administrations in Sprague-Dawley rats. Following IV administration, PEG-rhEPO exhibited about ~50% lower clearance than that of Aranesp®, leading to 50% higher systemic exposure than that of Aranesp®. PEG-rhEPO also showed a significantly longer $t_{1/2}$ than that of Aranesp® following IV administration (Fig. 7). However, following subcutaneous administration, Aranesp® appeared to have a better absorption, resulting in a better C_{max} and bioavailability than those of PEG-rhEPO, even though the systemic exposure was similar (Figs. 7 and 8).

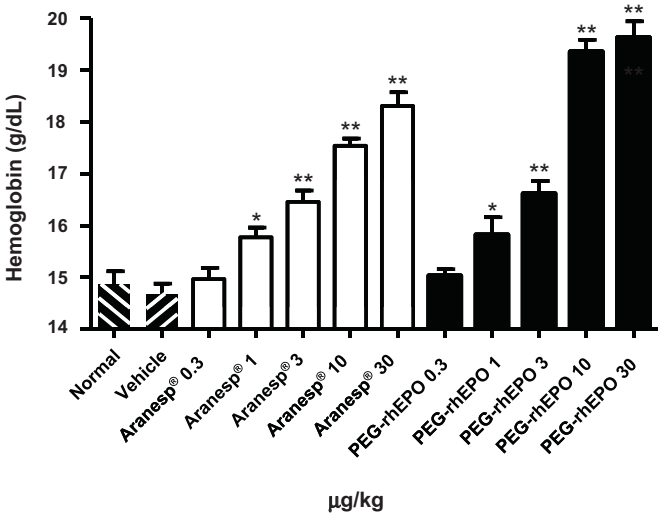
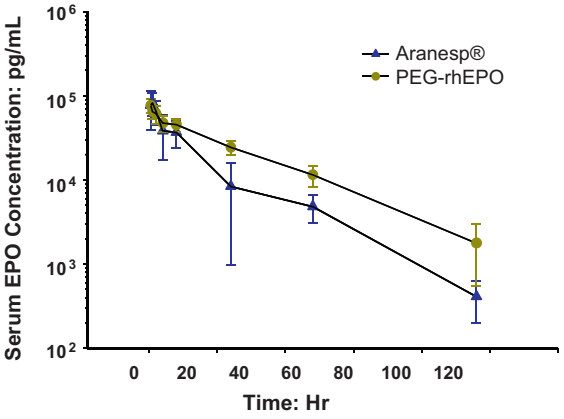
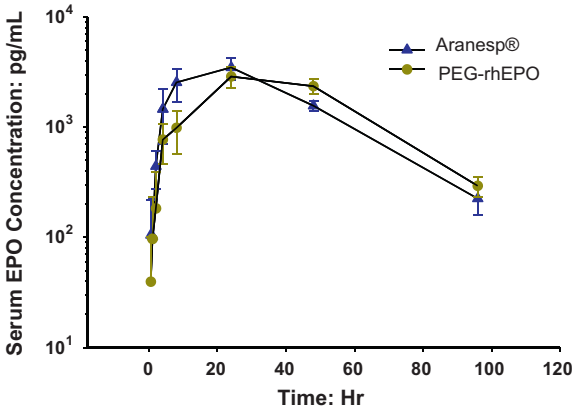


Fig. 6. Pharmacodynamic activity of PEG-rhEPO in mice is similar to Aranesp®. Groups ($n = 10$/group) of CD-1 mice were injected with various amounts of PEG-rhEPO or Aranesp® (between 0.3 and 30 μg/kg, molar equivalent based on protein mass) via IP route and hemoglobin levels were measured after 7 days. The data shown are group means of hemoglobin ± SEM. Statistical analyses were performed in GraphPad Prism. Significant difference from vehicle at $^{*}p < 0.05$, $^{**}p < 0.01$.



	Aranesp®	PEG-rhEPO
AUC $_{0-\infty}$ (ng/mL*hr)	1084±46	1656±330
CL (mL/hr/kg)	2.8±0.1	1.9±0.5
$t_{½}$ (hr)	13.6±1.4	23.3±5.2
V_{ss} (mL/kg)	55.3±5.6	55.8±15.5

Fig. 7. Pharmacokinetic comparisons of PEG-rhEPO and Aranesp® in rats following IV administration. Sprague-Dawley rats (4 males and 4 females per group) were dosed with PEG-rhEPO at 3 μg/kg or Aranesp® at 4.5 μg/kg through IV administration. Serum samples were obtained at 0.5, 1, 2, 4, 8, 24, 48 and 96 h post dose. The serum rhEPO concentrations were determined with Quantikine IVD human erythropoietin kits (R&D systems). The serum concentration of Aranesp® was normalized to 3 μg/kg dose for comparison to PEG-rhEPO group. PK parameters were derived by fitting concentration time data to NCA model in WinNonlin 5.01). Data expressed as Mean ± SD. $AUC_{0\text{-}INF}$, area under the serum concentration time curve from zero time to infinity. CL, clearance; $t_{1/2}$, terminal half-life; V_{ss}, volume of distribution at steady state.



	Aranesp®	PEG-rhEPO
AUC $_{0-\infty}$ (ng/mL*hr)	458±102	471±59
C_{max} (ng/mL)	10.5±2.4	8.8±1.7
T_{max} (hr)	24	24
Bioavailability (%)	33-52	25-32

Fig. 8. Pharmacokinetic comparisons of PEG-rhEPO and Aranesp® in rats following SC administration. Sprague-Dawley rats (4 males and 4 females per group) were dosed with PEG-rhEPO at 3 μg/kg or Aranesp® at 4.5 μg/kg through SC administration. Serum samples were obtained at 0.5, 1, 2, 4, 8, 24, 48 and 96 h post dose. The serum rhEPO concentrations were determined with Quantikine IVD human erythropoietin kits (R&D systems). The serum concentration of Aranesp® was normalized to 3 μg/kg dose for comparison to PEG-rhEPO group. PK parameters were derived by fitting concentration time data to NCA model in WinNonlin 5.01. Data expressed as Mean ± SD. $AUC_{0\text{-}INF}$, area under the serum concentration time curve from zero time to infinity; C_{max}, maximum serum concentration; T_{max}, time to maximum concentration.

4. Discussion

Although *P. pastoris*, in recent times, has become the organism of choice for the expression of a multitude of recombinant proteins (Cregg et al., 2009), this eukaryotic system has been hampered by the fact that glycoproteins when expressed are decorated by high-mannose yeast type glycans. It has been shown previously that glycoproteins with terminal mannose can be removed from the circulation due to specific interactions with mannose receptors. Proteins can be targeted to certain tissue types and organs which will result in reduced serum half life and undesired tissue distribution (Wileman et al., 1985; Solá and Griebenow, 2010). Yeast high mannose glycans are also potentially immunogenic in humans (Daly and Hearn, 2005). Earlier reports of rhEPO expression in wild type *P. pastoris* demonstrate that the main glycoform expressed in this unmodified host contains 17 mannose residues (Celik et al., 2007). Furthermore, a recent publication highlights the expression of rhEPO in wild type *P. pastoris* and shows that only PEGylated rhEPO has *in vivo* efficacy (Maleki et al., 2011). To overcome this problem, we have humanized the glycosylation machinery of this organism (Bobrowicz et al., 2004; Choi et al., 2003; Davidson et al., 2004; Hamilton et al., 2003, 2006; Nett et al., 2011) and show here that rhEPO produced in humanized *P. pastoris* is decorated with very homogeneous complex glycans and that PEG-rhEPO showed comparable PK and PD profiles to those of mammalian cell produced long acting Aranesp®.

It is well appreciated in the literature that changes in cell culture conditions can have a profound influence on recombinant protein production and quality (Hossler et al., 2009). In the case of rhEPO, unfavorable environments for glycosylation caused by stressful culture conditions can lead to a reduction in sialylated glycoforms (Yoon et al., 2004). Thus, while most of the secreted rhEPO from CHO cultures is biologically active, only a fraction of this material is considered suitable for use in pharmaceutical products. In addition, the choice of technology, for example roller bottles vs. hollow fiber reactors, can produce significant differences in glycosylation isoform composition for rhEPO produced in mammalian systems (Duvar et al., 2005; Yuen et al., 2003). These differences, and specifically the amount of sialylation, can then result in markedly altered *in vivo* and *in vitro* bioactivities. Our results on the other hand show that glycosylation fidelity of humanized *P. pastoris* is very well maintained in fermentation and downstream processes from as small as 5 ml to as large as 2000 l fermentation scales.

Another potential issue with non-human mammalian expression systems such as CHO cell lines lies in the fact that therapeutics produced in those systems are often decorated with the non-human sialic acid N-glycolylneuraminic acid (Neu5Gc) (Hokke et al., 1990; Noguchi et al., 1996). Until recently this contamination was generally ignored during drug development, because it had been reported that healthy individuals did not produce specific antibodies against Neu5Gc and it was therefore considered to be minimally immunogenic (Noguchi et al., 1996). Recent findings indicate, however, that all humans have variable amounts of Neu5Gc-specific circulating antibodies (Padler-Karavani et al., 2008; Tangvoranuntakul et al., 2003), and that these antibodies have the ability to interact and form immune complexes *in vitro* with certain biologics produced in non-human cell lines (Ghaderi et al., 2010). Non-humanized yeast cells also have the ability to add immunogenic glycans to the proteins they express. Although *P. pastoris* does not show the hyperglycosylation and terminal 1,3-mannose residues of *Saccharomyces cerevisiae*, it has the ability to add phosphomannose

and also β-linked mannose residues (Mille et al., 2008). In the case of *Candida albicans*, these beta-mannose residues in particular have been shown to be potent antigens for the adaptive immune response and to elicit specific antibodies (Han et al., 1997). In our case we identified an immunogenic glycan component in our initial expression strain. Although we had already knocked out the *BMT2* gene which is responsible for the majority of the β-linked mannose additions, the trace amounts of these apparently highly immunogenic glycans still gave rise to reactive antibodies in rabbits. However, knock-out of the remaining three BMT genes (*BMT1*, *BMT3*, and *BMT4*) in the final production strain then completely eliminated this immunogenic response (Hopkins et al., 2011).

EPO isolated from humans contains a single *O*-linked glycan at serine 126. It has been shown, however, that this glycan has no functional role, because EPO produced without *O*-linked carbohydrate exhibits normal *in vitro* and *in vivo* biologic activity and *in vivo* clearance (Wasley et al., 1991). The *P. pastoris* produced rhEPO in our studies had no detectable level of *O*-glycosylation and, in agreement with the above cited literature, no unusual *in vitro* or *in vivo* activity that could be attributed to this lack of occupancy.

Polyethylene glycols, or PEGs, are biocompatible polymers that, when conjugated with biopharmaceuticals such as proteins, peptides, antibody fragments and oligonucleotides, can improve the efficacy of a drug through increased bioavailability, decreased immunogenicity and decreased frequency of administration (Pasut and Veronese, 2009). The PEGylation of protein molecules can be achieved using several different chemistries. Sites of PEGylation on an amino acid sequence include N-terminal PEGylation specific to the α-amino group, thiol PEGylation specific to free cysteine, C-terminal carboxyl PEGylation and non-specific lysine PEGylation (Kozlowski and Harris, 2001). In our case we chose to PEGylate rhEPO at the N-terminus based on acceptable accessibility and reactivity of the latter. Mono PEGylated rhEPO was obtained in relatively high yields and separated from di-PEGylated protein using liquid chromatography to yield a highly homogeneous mono-PEGylated final product with predominantly N-terminal PEGylation.

rhEPO has a short *in vivo* half life and adding additional glycosylation sites and enriching sialylated (tetra- and tri-antennary) species greatly improve the half life of the molecule, exemplified by Aranesp®, although the modified EPO has about 4 fold lower *in vitro* binding activity. Humanized *P. pastoris* produced rhEPO possesses very homogenous bi-antennary glycans and the *in vitro* binding properties were shown to be better than that of Aranesp®. This bi-antennary rhEPO possessed a poor *in vivo* PK profile when dosed in rats (data not shown). However, when it was conjugated with PEG, its PK profile was greatly improved. Following IV administration, its systemic exposure was significant higher and the $t_{1/2}$ significantly longer than those of Aranesp® due to its significant lower clearance. Following SC administration, however, it appeared that Aranesp® was absorbed slightly better with higher C_{max} even though the systemic exposure was similar between the two. This seemingly paradoxical result may be explained by the better local absorption of Aranesp® but faster systemic clearance, leading to overall similar exposure after SC administration. The PD data in a mouse study are consistent with rat PK results in that the hemoglobin level was higher in animals treated with PEG-rhEPO than those treated with Aranesp® at the same dose levels.

Taken together, our results demonstrate that glycoengineered *P. pastoris* can successfully be used to produce rhEPO with highly homogeneous glycan structures, setting it apart from other, currently used production systems like CHO cells or wild type *P. pastoris*. Furthermore, this yeast based biologic shows comparable *in vitro* and *in vivo* characteristics to its counterparts produced in mammalian systems.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2011.11.002.

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