Appl Microbiol Biotechnol (2017) 101:1889–1898
DOI 10.1007/s00253-016-7973-y

Production process reproducibility and product quality consistency of transient gene expression in HEK293 cells with anti-PD1 antibody as the model protein

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Received: 23 August 2016 / Revised: 19 October 2016 / Accepted: 26 October 2016 / Published online: 16 November 2016
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Abstract Demonstration of reproducibility and consistency of process and product quality is one of the most crucial issues in using transient gene expression (TGE) technology for biopharmaceutical development. In this study, we challenged the production consistency of TGE by expressing nine batches of recombinant IgG antibody in human embryonic kidney 293 cells to evaluate reproducibility including viable cell density, viability, apoptotic status, and antibody yield in cell culture supernatant. Product quality including isoelectric point, binding affinity, secondary structure, and thermal stability was assessed as well. In addition, major glycan forms of antibody from different batches of production were compared to demonstrate glycosylation consistency. Glycan compositions of the antibody harvested at different time periods were also measured to illustrate N-glycan distribution over the culture time. From the results, it has been demonstrated that different TGE batches are reproducible from lot to lot in overall cell growth, product yield, and product qualities including isoelectric point, binding affinity, secondary structure, and thermal stability. Furthermore, major N-glycan compositions are consistent among different TGE batches and conserved during cell culture time.

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Keywords Transient gene expression · Production process reproducibility · Product quality consistency · Glycosylation conservation

Introduction

There are a growing number of therapeutic biological molecule candidates in the pipeline waiting for preclinical and clinical evaluation. As the medical treatment is on its way to precision medicine, it will bring us much more candidates to evaluate in development stages. Referred to the DrugBank database, it contains over 6000 experimental drugs and 4331 non-redundant protein (i.e., drug target/enzyme/transporter/carrier) sequences are linked to these drug entries (DrugBank 2016). Despite a significant and continuous increase in medical research spending, the number of new drugs approved and new drug targets identified each year have remained almost constant for the past 20–25 years, with about 20 new drugs and 5 new targets being approved worldwide each year. At this rate, it would take many years to evaluate the existing candidates in clinical development according to current technology (Zhu 2013). Besides, manufacturing of biological materials is quite resources consuming while only 13.2% (Hay et al. 2014) of the large molecule candidates entering phase 1 clinical trials can be on the market in the end, which makes the cost for the development of innovative biological drugs extremely high. So, if one can establish a procedure to screen the candidates with a shorter period of time and lower cost, it would dramatically facilitate the development of innovative biological therapeutics.

Mammalian cells have been used as an expression system for many recombinant therapeutic proteins (Zhu 2012). Traditionally, generating cell lines to express recombinant proteins usually takes 6 to 12 months (Zhu 2013). Transient

gene expression (TGE) is the expression of a gene of interest for a short-defined period of time after transfection without being stably integrated into the host chromosomes, which only takes several weeks to set up a production system. Compared to stable gene expression (SGE), the upstream process of TGE is much simpler (Fig. 1a), involving only one step of transfection of cells from an established cell bank. This eliminates cell line development and cell line stability assessment in SGE (Fig. 1b) including stable pool selection, single clone selection, clone characterization in cell growth and product expression, as well as cell banking. So, obviously, TGE takes much less resources to generate products than SGE does.

Over the last decade, a TGE method has been actively developed. A wide range of products from monoclonal antibody (Backliwal et al. 2008a; Rajendra et al. 2011), Fc fusion protein (Suen et al. 2010; Zustiak et al. 2014), to other recombinant proteins (Baldi et al. 2005; Cho et al. 2003; Jiang et al. 2015) have been successfully manufactured by a TGE system with high product quality. The production of TGE has been scaled up to 100 l (Girard et al. 2002), and the expression level reached 1 g/l for monoclonal antibody (mAb) (Backliwal et al. 2008a). Technically, it is feasible now to produce 100 g of protein with reasonable similar glycan composition by TGE, which were demonstrated by several publications (Galbraith et al. 2006; Nallet et al. 2012; Ye et al. 2009). However, TGE has not been accepted for manufacturing therapeutic materials for a preclinical toxicology study and early human clinical trials, except for vaccine products (Gutierrez-Granados et al. 2016; Harper et al. 2004). Production process reproducibility and product quality consistency using TGE have been the main concerns. In this research, we evaluated the cell growth, cell apoptotic status, and multiple batch product yield and quality profile through physicochemical and biological activity assays, as well as analysis of glycan composition.

Materials and methods

Plasmid construction

The plasmid (pM09) featuring the EBoriP origin for plasmid replication in human embryonic kidney 293 (HEK293) cells and the CAG promoter for driving transgene expression was used for the expression of anti-programmed cell death protein 1 (PD1) (GenBank AAC51773.1) mAb (Korman et al. 2006). The plasmid and DNA fragment encoding anti-PD1 mAb were both digested with *Hin*dIII and *Bam*HI restriction endonuclease (TaKaRa Biotechnology, Dalian, Liaoning, China) and then ligated with solution I (TaKaRa Biotechnology,

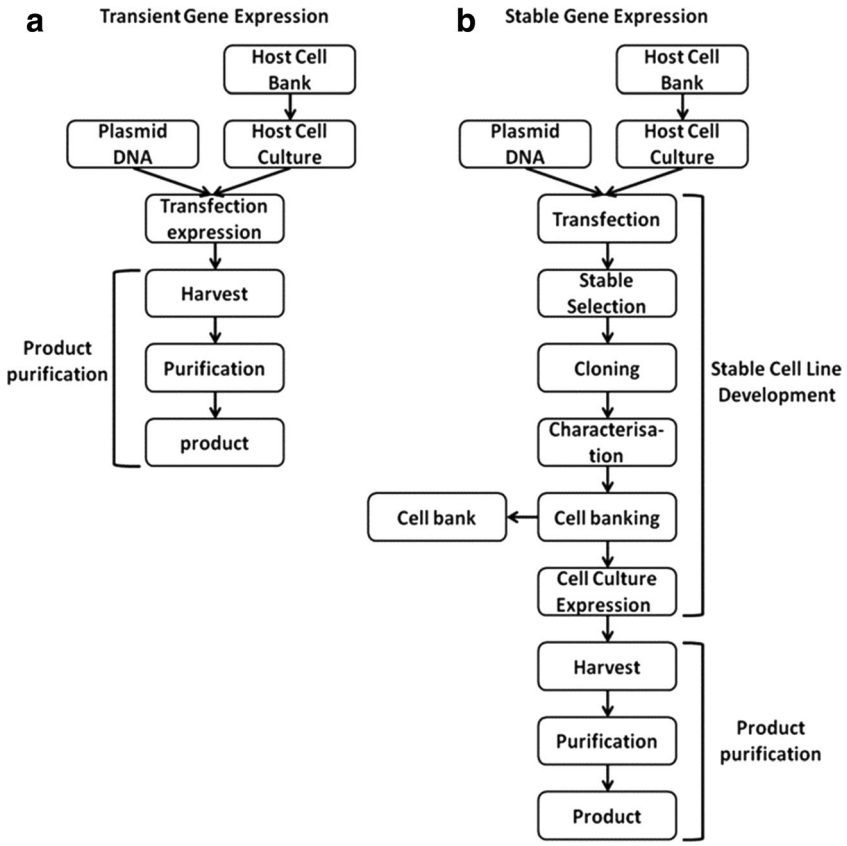

Fig. 1 Process flowcharts for transient (**a**) and stable (**b**) gene expression (Zhu 2013)

Dalian, Liaoning, China). The heavy chain and light chain were inserted into the plasmid separately.

Cell culture and transfection

Cell lines tested for TGE included HEK293 (HEK293E; ATCC, USA) and Chinese hamster ovary (CHO) (CHO-S; Invitrogen, Carlsbad, CA, USA) cells. HEK293 cells were grown in suspension in a serum-free freestyle F17 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 8 mM GlutaMAX (Invitrogen, Carlsbad, CA, USA), 0.1% Pluronic F68 (Invitrogen, Carlsbad, CA, USA), and 25 μg/ml Geneticin (Invitrogen, Carlsbad, CA, USA). CHO cells were grown in suspension in a serum-free circular dichroism (CD)-CHO medium (Invitrogen, Carlsbad, CA, USA) supplemented with 8 mM GlutaMAX (Invitrogen, Carlsbad, CA, USA). Both cells were cultured in disposable polycarbonate erlenmeyer flasks (Corning, New York, NY, USA), shaken at 130 rpm in a humidified incubator at 37 °C in the presence of 5% CO_2, and were resuspended in a fresh medium every 3–4 days at a concentration of 0.5×10^6 cells/ml.

Linear 25 kDa polyethylenimine (PEI) (Polysciences, Warrington, PA, USA) with a concentration of 1 mg/ml was used as the transfection agent. According to the literature (Raymond et al. 2011), 2 days before transfection, HEK293 cells were seeded at 0.5×10^6 cells/ml. On the day of transfection, the cell density was $1.5–2.0 \times 10^6$ cells/ml. One microgram of plasmid DNA (Lc/Hc = 2:1, *w*/*w*) was used per milliliter of transfection volume. The DNA was diluted with a freestyle F17 medium containing all the supplements to a concentration of 40 ng/μl. Then, PEI (DNA/PEI = 1:3, *w*/*w*) was added to the diluted DNA. After incubation at room temperature for 10–15 min, the DNA and PEI mixture was added to the cell culture. Twenty-four hours post transfection, 20% (*w*/*v*) tryptone N1 (Organotechnie S.A.S, 27, Avenue Jean Mermoz, 93120, La Courneuve, France) was added to the culture to a final concentration of 0.5% (*w*/*v*). CHO cells were transfected at a density of 3×10^6 cells/ml with 4 μg DNA (Lc/Hc = 4:1, *w*/*w*) and 8 μg PEI per milliliter of transfection volume. Normally, except indicated specifically, 4 h post transfection, 0.5 mM valproic acid (VPA) (Sigma-Aldrich, Epalinges, Switzerland) was added to the culture (Backliwal et al. 2008b) and the cells were shifted to 31 °C (Wulhfard et al. 2008). The culture was harvested when the cell viability decreased to 50%.

Cell viability measurement and apoptosis analysis

Viable cell density and cell viability were measured with a trypan blue staining method. Cells were stained with trypan blue (1:1, *v*/*v*; AMRESCO, Solon, OH, USA) and counted by the cell counter (Ruiyu Biotech, Shanghai, China). For apoptosis analysis, cells were stained with an annexin V-FITC/propidium iodide (PI) kit (Vazyme Biotech, Nanjing, China) according to the manufacturer's manual and detected by the flow cytometry BD LSRFortessa (BD Biosciences, San Jose, CA, USA).

Quantitation of human IgG antibody in cell culture supernatant by ELISA

A sandwich ELISA (de la Cruz Edmonds et al. 2006) was established to quantitate the concentration of antibody in cell culture supernatant. Briefly, a 96-microwell plate (Jet Biofil, Guangzhou, China) was coated with monoclonal anti-human κ chain antibody (Merck Millipore, Shanghai, China) for the capture of the antibody from the supernatant and then a monoclonal HRP-anti-human IgG antibody (F_C specific; Jackson ImmunoResearch, West Grove, PA, USA) was used for detection. TMB, a chromogenic substrate, was added for enzymatic oxidation of HRP, resulting in an intensity change detectable by a spectrophotometer at 450 nm. A seven-point standard curve from approximately 8 to 250 ng/ml was used to calculate the concentrations.

Product purification by protein A

The product (anti-PD1 mAb) was purified by protein A chromatography. A prepacked HiTrap rProtein A FF column (GE Healthcare Life Sciences, Beijing, China) was used for the purification of the antibody. The column was equilibrated with five column volumes (CVs) of binding buffer (20 mM (pH 7.2) phosphate buffer containing 150 mM NaCl) and eluted with three column volumes of elution buffer (100 mM (pH 3.0) citric acid buffer). The elution was adjusted to pH 7.0 with 1 M (pH 9.0) Tris-HCl buffer.

Isoelectric point measurement

The isoelectric point (PI) of the product was measured by isoelectric focusing electrophoresis (IEF). Two micrograms of the purified antibody was loaded onto the IEF gel (Invitrogen, Carlsbad, CA, USA). The run condition was 100 V constant for 1 h, 200 V constant for 1 h, and 500 V constant for 30 min. The IEF gel was fixed in 12% (*v*/*v*) trichloroacetic acid (TCA) containing 3.5% (*v*/*v*) sulfosalicylic acid for 30 min and stained using a collodial blue staining kit (Invitrogen, Carlsbad, CA, USA).

Binding affinity comparison

Binding affinity of anti-PD1 antibody to PD1-His protein consisting of the extracellular domain of PD1 (amino acids 21–170) and six-histidine protein at the C terminal of PD1 was measured using biolayer interferometry (Abdiche et al. 2008). Analysis was performed on the OctetRed device

(ForteBio, Inc., Menlo Park, CA, USA) at 30 °C with an agitation of 1000 rpm. Samples or buffer was dispensed into 96-well plates (Merck Millipore, Shanghai, China) at a volume of 200 μl. Ni-NTA biosensor tips (ForteBio, Inc., Menlo Park, CA, USA) were prewet in phosphate-buffered saline (PBS) which serves as the background buffer and then were immobilized with 100 μg/ml PD1-His protein. After a wash in PBS, the tips were exposed to 200 μg/ml anti-PD1 antibody for association step and transferred to PBS for dissociation step afterwards. Duplicate biosensor tips without immobilization with PD1-His protein were used as control. Corrected data were analyzed using the ForteBio data analysis software.

Secondary structure comparison

Secondary structure of the product was compared by a CD spectrum. Purified antibody was dissolved in 5 mM phosphate buffer (pH 7.4) at a concentration of 200 μg/ml and measured with a JASCO J-815 CD spectrometer (JASCO, Tokyo, Japan) with a path length of 0.1 cm at room temperature. The CD spectra were collected from 190 to 250 nm with a bandwidth of 1.0 nm, a scanning speed of 50 nm/min, and an accumulation of two scans. All the spectra were corrected from the contribution of the buffer.

Thermal stability measurement

Thermal stability of the product was measured by differential scanning fluorimetry (DSF) (Alexander et al. 2014) using the Prometheus NT.48 instrument (NanoTemper Technologies, Munich, Germany). For this, 10 μl of 200 μg/ml antibody was loaded onto nano-DSF grade standard capillaries. Thermal unfolding of the antibody was analyzed in a thermal ramp from 20 to 95 °C with a heating rate of 1 °C/min.

Glycosylation analysis by HPLC

A HPLC-based method was applied to analyze the glycosylation of the product. Two hundred fifty micrograms of the purified antibody was digested with 2 μl PNGaseF (NEB, Beijing, China) at 37 °C overnight to release the N-linked oligosaccharides from the antibody. A cold ethanol precipitation method was used to eliminate the protein from the digest. The supernatant was taken out and dried using a centrifugal evaporator (Thermo Fisher Scientific, Shanghai, China). Five microliters of 2-aminobenzamide (2-AB) labeling reagent (ProZyme, CA, USA) was added to the dried N-glycan sample. The mixture was incubated at 65 °C for 3 h. An excess labeling reagent was removed by cartridges (ProZyme, CA, USA) according to the manufacturer's manual. The purified 2-AB-labeled N-glycans were dried using a centrifugal evaporator and redissolved in 50 μl of 60% acetonitrile in water (*v*/*v*) for HPLC analysis.

The 2-AB-labeled N-glycans were separated on a SeQuant® ZIC®-HILIC column (5 μm, 250 × 4.6 mm) from Merck, and the LC system was Agilent 1260 (Agilent, Beijing, China). Mobile phase A was 10 mM NH_4AC in 80:20 acetonitrile (ACN)/water (*v*/*v*) and B was 10 mM NH_4AC in 50/50 ACN/water (*v*/*v*). The column was equilibrated with 80% phase A overnight. The flow rate was 1 ml/min, and the temperature of the column compartment was 35 °C. Five microliters of the sample was injected, and the gradient was evaluated as follows: 0 min, 20% B; 10 min, 35% B; 35 min, 60% B; 36 min, 100% B; 42 min, 100% B; and 43 min, 20% B, which was kept for 17 min for column equilibration. The analytes were detected by a fluorescence detector with an excitation wavelength of 330 nm and an emission wavelength of 420 nm.

Results

Optimization of PEI-mediated transfection of HEK293 cells

By means of expressing enhanced green fluorescent protein (EGFP) as the reporter protein, PEI-mediated transfection efficiency of HEK293 cells under different concentrations of plasmid and PEI was measured. Three days post transfection, cells were harvested and resuspended in PBS and detected by the flow cytometry BD LSRFortessa with blank plasmid-transfected cells as the negative control (Fig. 2). A DNA concentration of 1 μg/ml with a DNA/PEI (*w*/*w*) ratio of 1:3 obtained the highest transfection efficiency, which was about 60%.

Assessment of the reproducibility of TGE process

Nine batches of production by HEK293 cells were conducted to assess the reproducibility of TGE process. Viable cell density, cell viability and cell apoptotic status during expression, and the antibody yield in cell culture supernatant were compared among different batches. On the same day post transfection, viable cell density and cell viability variance among the nine batches were 4.57–9.95 and 1.88–8.62% during a 6-day culture (Fig. 3a). The ratio of FITC−/PI− and FITC+/PI+ cells to total cells varied by 1.06–13.27 and 2.47–6.48% among the nine batches (Fig. 3b). Antibody yield was about 90 mg/l with a relative standard deviation (RSD) of 11.82% (Fig. 3c). As it is concluded from the results, the TGE process was reproducible in overall cell growth and products by the process were yielded in a relatively narrow quantity range (Fig. 3).

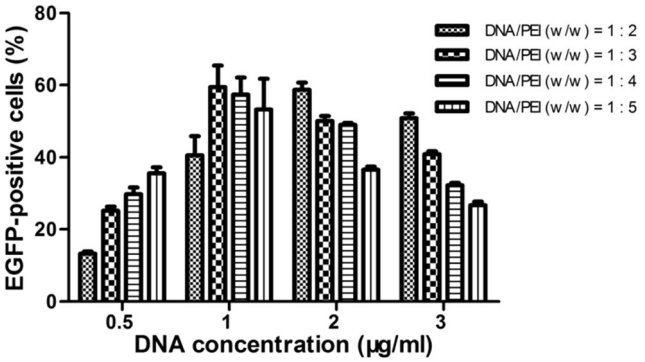

Fig. 2 Transfection efficiency of HEK293 cells under different concentrations of plasmid and PEI. *Error bar* represents the standard deviation (SD) of at least two independent transfections

Assessment of the quality consistency of the products

The quality consistency of the product from nine batches of production by HEK293 cells through TGE was assessed by analyzing physicochemical and biological properties including isoelectric point (PI), binding affinity, secondary structure, and thermal stability.

The PIs of the nine batches of the antibody were compared by isoelectric focusing electrophoresis (IEF) as described in the "Materials and methods" section. As shown in Fig. 4a, all these products (anti-PD1 mAb) showed a similar band between pH 7.8 and 8.0. Besides of the main band, there were several light bands in the IEF gel, which might be caused by the variation of glycosylation of the antibody. Affinity profiles of the products from the nine-batch production were compared using biolayer interferometry (Fig. 4b). All products had a similar K_D between 1.73 and 1.82 nM with a RSD of 1.56% (Fig. 4c). Secondary structure of the antibody was compared using circular dichroism (CD) spectrum. As shown in Fig. 4d, the CD spectra of all the products were similar, which indicated that the secondary structure of the antibody was comparable. Thermal stability of the antibody was measured by nano-DSF as described in the "Materials and methods" section. As shown in Fig. 4e, f, products from different batches had quite similar onset temperature around 55 °C and melting temperature around 61 °C, indicating that thermal stability of the antibody from different production batches was in a narrow range. According to these data, quality attributes of the antibody from different TGE batches were consistent in PI, binding affinity, secondary structure, and thermal stability, demonstrating highly batch-to-batch consistent product quality from production using the TGE process.

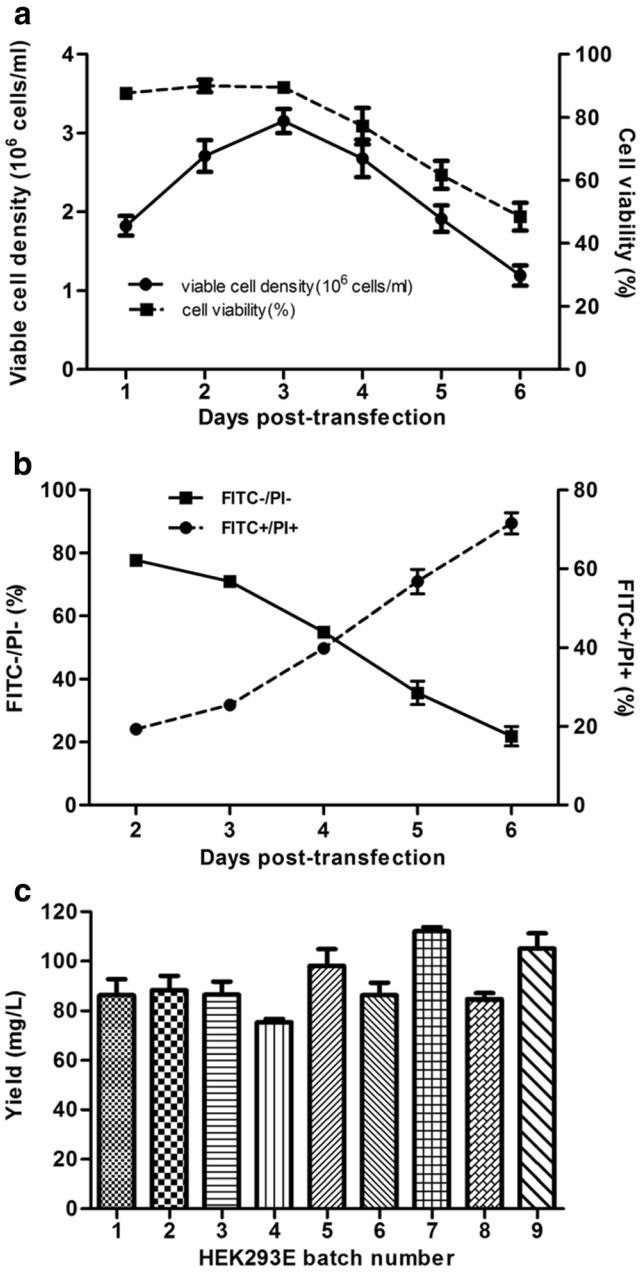

Fig. 3 Comparison of cell growth and productivity of different TGE batches. **a** Viable cell density (*full line*) and cell viability (*dotted line*) measured by a cell counter using a trypan blue staining method. The data is the mean of nine batches ± SD. **b** Cell apoptotic status measured by flow cytometry using an annexin V-FITC/PI staining method. The data is the mean of nine batches ± SD. **c** Antibody yield in the cell culture supernatant of HEK293 cells measured by ELISA. The *error bar* represents at least three technical replicates

N-Glycosylation analysis of products from different TGE batches

Glycosylation of a biopharmaceutical product is closely associated with cell line and production process. Therefore, a glycan pattern of the products from different TGE production batches reflects the consistency of post-translational modification of the product through TGE. A HPLC-based method was applied to characterize N-glycosylation of the antibody products transiently expressed in HEK293 cells as described in the "Materials and methods" section. Figure 5a shows the reducing SDS-PAGE analysis of the nine batches of the

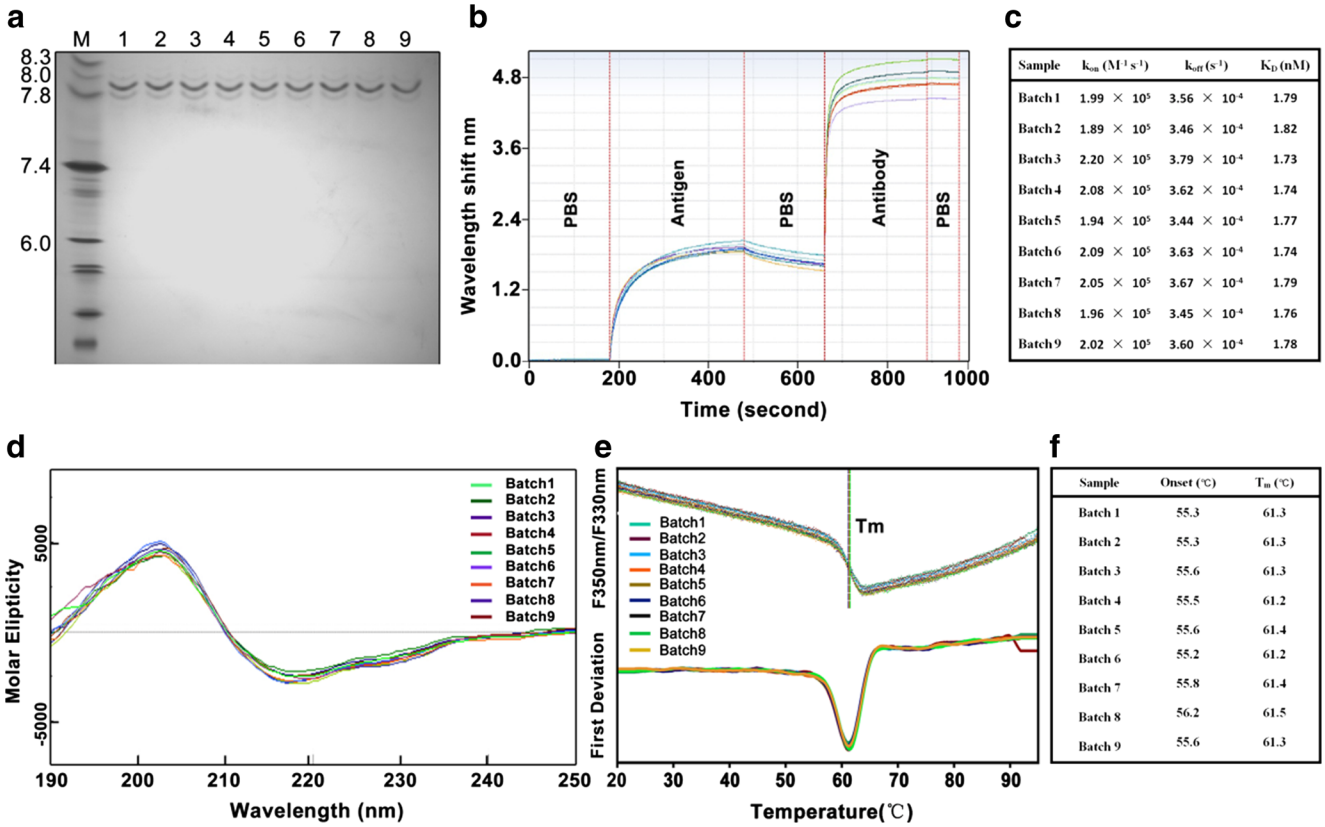

Fig. 4 Comparison of physicochemical and biological properties of products from different batches of production by HEK293 cells. **a** IEF of nine batches of the antibody. *Lines 1–9* represent batch numbers 1–9, respectively. **b** Process data of biolayer interferometry. The *notes in the figure* represent the solution that biosensor tips were kept in at different steps. **c** K_{on}, K_{off}, and K_D of nine batches of the antibody measured by biolayer interferometry using an OctetRed instrument. **d** Circular dichroism spectra of nine batches of the antibody collected from 190 to 250 nm. **e** DSF measurement of nine batches of the antibody using a Prometheus NT.48 instrument. **f** Onset temperature and T_m of nine batches of the antibody

antibody before and after PNGaseF digestion. Each heavy chain showed a similar molecular weight and reduced molecular weight after the digestion. The major N-glycans of the antibody (G0, G0F, G1, G1F, and G2F) were well separated on SeQuant® ZIC®-HILIC column (Fig. 5b, c), which was described in the "Materials and methods" section. The relative abundance of the glycans (Table 1) was calculated based on the corresponding peak area. G2 was below the limit of detection of the method. All these nine batches of the antibody showed a similar glycan distribution (Fig. 5d). The RSD of G0, G0F, G1, G1F, and G2F was 13.22, 4.17, 13.92, 2.38, and 7.39%, respectively. The higher RSD of G0 and G1 was probably due to their low abundance, which made it hard for accurate measurement.

As glycosylation may reflect on in vivo metabolic profile, consistent glycan pattern has been interpreted as one of key parameters in the assessment of lot-to-lot consistency of the products. Therefore, we try to assess the glycosylation consistency with production using another cell line CHO cells. Similar to the result as observed in HEK293 cells, all eight batches of the antibody expressed in CHO cells showed a similar glycan distribution (Fig. 5e). The RSD of G0, G0F, G1, G1F, and G2F was 8.88, 1.17, 11.27, 2.83, and 5.99%, respectively. In addition, N-glycan distribution of the antibody from HEK293 cells and CHO cells was compared to determine whether glycan composition is cell line dependent. As shown in Fig. 5f, antibody from HEK293 cells had a higher ratio of G1F and G2F than that from CHO cells. According to the data above, the glycan composition of the antibody produced by TGE is consistent among different batches but varies between HEK293 and CHO cell lines.

To determine whether the N-glycan distribution varied with time during cell culture, we characterized N-glycans of the antibody from the same batch of production by HEK293 cells harvested at 3 (D3), 5 (D5), 7 (D7), and 11 (D11) days post transfection. All supernatants were taken out, and cells were resuspended in a fresh medium. As shown in Fig. 6, despite that cell viability was decreasing with the time during cell culture, the N-glycan distribution of D3, D5, D7, and D11 was similar and the RSD of main glycoforms G0F, G1, G1F, and G2F was 9.25, 17.34, 6.83, and 18.90%, respectively.

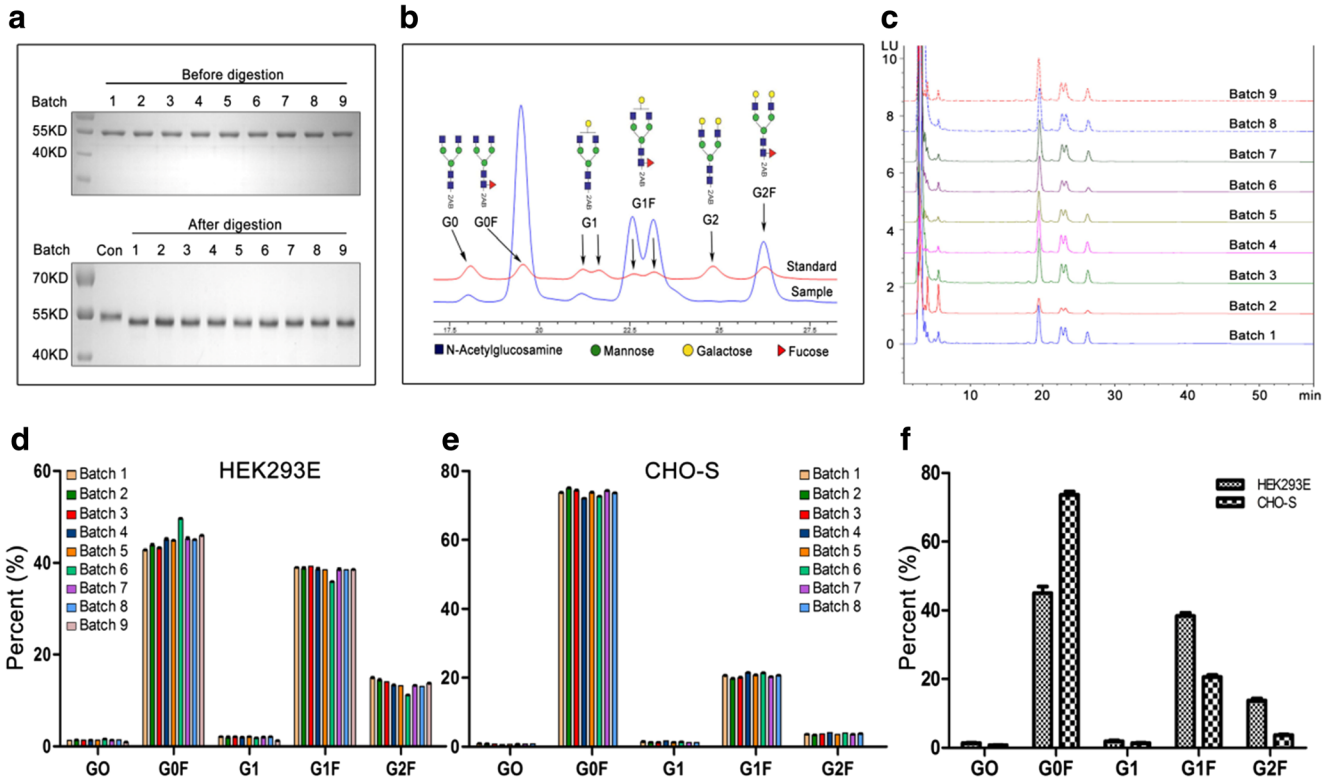

Fig. 5 Glycosylation analysis of products from different batches of production. **a** Reducing SDS-PAGE analysis of IgG both before and after the digestion of PNGaseF. *Numbers 1–9* represent batches 1–9. **b**, **c** Hydrophilic HPLC chromatography of 2-AB-labeled N-glycans. **d** Relative abundance of major glycans of the antibody transiently expressed in HEK293 cells. *Error bar* represents the SD of at least two technical replicates. **e** Relative abundance of major glycans of the antibody transiently expressed in CHO cells. *Error bar* represents the SD of at least two technical replicates. **f** Comparison of the relative abundance of major glycans of the antibody transiently expressed in HEK293 cells and CHO cells. The data is the mean of nine batches ± SD for HEK293 cells and the mean of eight batches ± SD for CHO cells

Discussion

There are thousands of cytokines, mAbs, and recombinant proteins in preclinical to early clinical development worldwide, particularly in precision medical treatment or personalized drug development. A simple way to accelerate the preclinical and clinical development of innovative biopharmaceuticals would be greatly appreciated. TGE has been critically pursued to serve for the purpose. However, demonstrating reproducibility and quality consistency of the product by the TGE production process is a challenging mission for industry to utilize this technology for manufacturing materials for IND-driven toxicology study and clinical trials. There were several reliable results showing that TGE production may produce high-quality biopharmaceuticals and quality attributes including glycan compositions were reasonably stable (Galbraith et al. 2006; Nallet et al. 2012; Ye et al. 2009). Nevertheless, systematic investigations on evaluating TGE production process and its product quality have not been found in publications. We believe that monitoring production process closely and evaluation of quality aspects of the final products manufactured by the TGE may provide reliable evaluation of the applicability of the method in the industrial development of biotherapeutics. In this study, we tried to produce the model antibody (anti-PD1 mAb) under repeated cultural conditions and parameters with focus on product quality. To effectively reach meaningful product expression, we first optimized the TGE conditions including ratios of key reagents in order to improve the expression of the mAb in HEK293 cells. Then, nine batches of production in HEK293 cells were conducted using TGE in a non-instrumental cultivation system. Process-related parameters such as cell density, cell viability, and cell apoptosis assessed during cultural time after TGE showed process reproducibility with narrow variation in overall cell growth (Fig. 3a, b), resulting in reproducible product expression (Fig. 3c). More critical aspects are product quality attributes such as PI, binding affinity, secondary structure, and thermal stability which were compared and showed excellent comparability among multiple lots. The most critical quality attributes, N-glycosylation of the IgG antibody from different TGE batches, were also compared to demonstrate the comparability in post-translational modification.

According to the guideline of European Medicine Agency (EMA 2014), the PI, binding affinity, secondary structure, and thermal stability are all important profiles required to be compared among products from different batches to demonstrate

Table 1 The N-glycan distribution of the antibody from different batches of production using TGE

Batch	G0 (%)	G0F (%)	G1 (%)	G1F (%)	G2F (%)
H1	1.33	42.71	2.11	39.00	14.84
H2	1.40	43.73	1.87	38.70	14.30
H3	1.38	43.24	2.06	39.29	14.02
H4	1.36	44.96	1.93	38.52	13.22
H5	1.39	44.84	2.11	38.48	13.18
H6	1.57	49.59	1.82	35.90	11.12
H7	1.37	45.12	1.95	38.41	13.15
H8	1.42	44.98	2.10	38.48	13.03
H9	0.87	45.82	1.21	38.45	13.63
C1	0.82	73.66	1.40	20.52	3.60
C2	0.82	74.94	1.27	19.62	3.35
C3	0.75	74.32	1.26	19.99	3.68
C4	0.65	72.09	1.72	21.47	4.08
C5	0.71	73.70	1.28	20.73	3.57
C6	0.70	72.63	1.41	21.29	3.97
C7	0.75	74.22	1.25	20.22	3.55
C8	0.86	73.53	1.23	20.63	3.75
D3	0.27	40.89	1.36	44.03	13.45
D5	0.57	52.75	1.27	36.96	8.45
D7	0.45	50.20	1.59	38.56	9.20
D11	0.34	49.58	1.96	38.40	9.71

H1 represents HEK293 batch 1, C1 represents CHO batch 1, and D3 represents the product harvested at day 3 post transfection

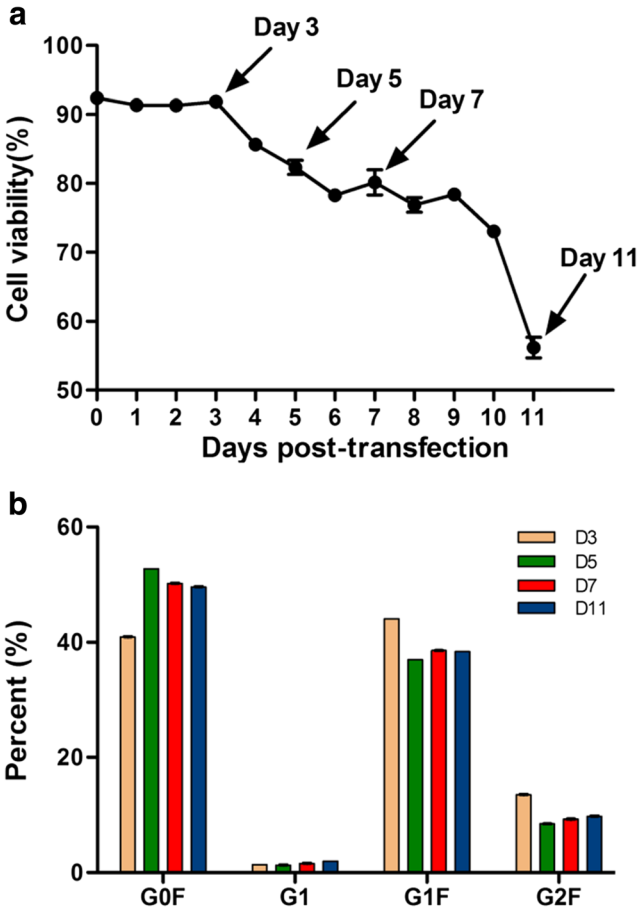

Fig. 6 Glycosylation analysis of products from the same batch of production by HEK293 cells harvested at different time periods. **a** Cell viability of HEK293 cells during an 11-day culture measured by a cell counter using a trypan blue staining method. *Error bar* represents the SD of at least three technical replicates. **b** Relative abundance of major glycans of products harvested at 3, 5, 7, and 11 days post transfection in HEK293 cells. *Error bar* represents the SD of at least two technical replicates

the product quality consistency. PI of the antibody has an effect on its pharmacokinetics, which, in result, may influence the dose, dose frequency, efficacy, and safety of the drug (Igawa et al. 2010; Lee and Pardridge 2003). Antibody binding affinity may impact its tumor targeting and penetration (Rudnick et al. 2011). High binding affinity will limit the diffusion of the antibody into tumors because slow rates of dissociation decrease the concentration of local diffusible, free antibody, while low binding affinity will decrease its targeting ability. Proper folding of the antibody is the base of its activity, and elevated temperature is probably the most common stress to cause the structure of the antibody to be lost as well as the bioactivity. As Fig. 4 shows, the PI, binding affinity, secondary structure, and thermal stability of these nine batches of the antibody are all highly similar, which indicate that the product qualities of the antibody from different TGE batches are reasonably stable.

Glycosylation is vital for the biological function and structure of human immunoglobulins. The glycans provide recognition epitopes and maintain the structure and stability of the immunoglobulins (Arnold et al. 2007; Jefferis 2005). They can impact effector functions such as antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) (Arnold et al. 2007; Jefferis 2005). Damen et al. (2009) had compared the N-glycan distribution of different batches of a commercial antibody trastuzumab, and the RSD of G0, G0F, G1F, and G2F among four batches was 25.16, 14.77, 8.16, and 27.96%, respectively. The N-glycan distribution of another commercial antibody bevacizumab was also compared, and the RSD of G0, G0F, G1, G1F and G2F among four batches was 3.17, 4.53, 21.34, 18.61, and 25.76%, respectively (Toyama et al. 2012). In this study, the N-glycan distribution variance of the antibody produced by TGE is very similar to that of different lots of commercial antibody produced by SGE. In addition, it is confirmed by our results that the glycan composition is cell line dependent between HEK293 cells and CHO cells (Fig. 5f), which is agreeable to the observation as Ye et al. (2009) reported. We further evaluated the glycan distribution variance over the HEK293 cell culture time (Fig. 6) and confirmed that as the cell viability decreased over the time, it did not cause significant alteration

in glycan distribution of the antibody. The same result was also observed in CHO cells (Galbraith et al. 2006).

The volume of TGE at 100 ml in this study was still not at industrial scale. However, as only one medium was used for both transfection and cell growth in this study and TGE system scaled up to 100 l has been repeatedly reported (Girard et al. 2002; Muller et al. 2007; Tuvesson et al. 2008), engineering scale-up to pilot scale can be expected with reproducible results.

The results of this study provide a convincing conclusion to the biopharmaceutical industry and the regulatory agencies that the products obtained via TGE are acceptable in product quality consistency and reproducibility which are the bases for future regulatory approval for early-phase clinical trials. This will significantly accelerate the development of innovative biologics and pharmaceuticals with a cost-effective method and find effective personal medication from thousands of candidates within a relatively short time period.

Acknowledgments The project was supported by the Science and Technology Commission of Shanghai (Grant Nos. 15DZ0503700 and 14712400300) and partially supported by the Natural Science Foundation of China (Grant No. 81502969).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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