

Different fermentation processes produced variants of an anti-CD52 monoclonal antibody that have divergent in vitro and in vivo characteristics

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Abstract The anti-CD52 antibody has already been approved for the treatment of patients with resistant chronic lymphocytic leukemia, relapsing-remitting multiple sclerosis, and has demonstrable efficacy against stem cell transplantation rejection. A CHO cell line expressing a humanized anti-CD52 monoclonal antibody (mAb-TH) was cultivated in both fed-batch and perfusion modes, and then purified. The critical quality attributes of these mAb variants were characterized and the pharmacokinetics (PK) properties were investigated. Results showed that the perfusion culture achieved higher productivity, whereas the fed-batch culture produced more aggregates and acid components. Additionally, the perfusion culture produced similar fucose, more galactose and a higher proportion of sialic acid on the anti-CD52 mAb compared to the fed-batch culture. Furthermore, the perfusion process produced anti-CD52 mAb had higher complement-dependent cytotoxicity (CDC) efficacy than that produced by the fed-batch culture, a result probably linked to its higher galactose content.

However, antibody produced by fed-batch and perfusion cultures showed similar PK profiles in vivo. In conclusion, perfusion is a more efficient method than fed-batch process in the production of functional anti-CD52 monoclonal antibody. Product quality variants of anti-CD52 mAb were found in different cell culture processes, which demonstrated different physiochemical and biological activities, but comparable PK properties. Whether these observations apply to all mAbs await further investigation.

Keywords Antibody · Process · Quality · Heterogeneity · Pharmacokinetics

Introduction

The CD52 antigen is a lipid-anchored glycoprotein expressed on the surface of lymphocytes, monocytes, and macrophage at

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a relatively high density but not on hematopoietic stem cells, making it a promising target for lymphoma therapy and immune suppression (Hale 2001). The anti-CD52 antibody has already been approved for the treatment of patients with resistant chronic lymphocytic leukemia (under the trade name Campath), relapsing-remitting multiple sclerosis (under the trade name Lemtrada), and has demonstrable efficacy against stem cell transplantation rejection (Holgate et al. 2015). During the cell culture process development of a biosimilar of the Alemtuzumab, a choice must be made between a fed-batch and a perfusion production mode, because these processes may affect product quality, drug efficacy, and possibly safety (Hu and Aunins 1997; Lee et al. 2012).

Fed-batch and perfusion cell cultures are the two principal modes of large-scale industrial production of recombinant therapeutic proteins (Butler 2005; Kretzmer 2002; Siganporia et al. 2014; Yang et al. 2007). In perfusion mode, media with products is perfused through the fermenter, while cells are retained or recycled back. For the production of unstable therapeutic proteins such as recombinant enzymes and blood coagulation factors, the perfusion process is much more suitable. It offers consistent and steady culture conditions, allows rapid removal of product, and yields stable and uniform product of high quality (Choo et al. 2007; Komolpis et al. 2010; Konstantinov and Cooney 2015; Tang et al. 2007). However, the long culture duration and the implication of cell retention devices increase the complexity of operation and process development. The fed-batch process is much more robust, in which feed nutrients and buffer are added at expected intervals to the bioreactor, and the products are harvested at the end of the run. The fed-batch process has dominated the production of monoclonal antibody products, except for a few such as ReoPro, Remicade, Simulect, Simponi, and Stelara were produced by the perfusion process (Pollock et al. 2013; Siganporia et al. 2014). Nevertheless, in order to meet the batch productivity, an extremely large cell culture vessel is needed in the fed-batch process, which leads to high infrastructure investment as well as low flexibility of the facility.

Monoclonal antibodies are large molecular weight glycoprotein with highly complicated and dynamic high order structure. Several groups have reported that process change can affect quality attributes including aggregates, charge heterogeneity, glycan distribution, and influence the protein drug efficacy and serum half-life. In this study, we established fed-batch and perfusion processes to culture Chinese hamster ovary (CHO) cells to produce anti-CD52 antibody and then characterized the critical quality attributes of these variant products, assessed whether the variants had different product efficacy and pharmacokinetic (PK) properties. Similar culture parameters were utilized in both culture modes in order to perform a head-to-head comparison. The product quality was assessed by comparing the purity, aggregates, charge heterogeneity, post-translational modification, and potency between the fed-batch and perfusion cultures.

Another important part of drug development is the measurement of PK properties *in vivo*, which might be affected by the *in vitro* quality of the product. Thus, we also carried out a PK bio-comparability study in cynomolgus monkeys.

Materials and methods

Cell culture

The anti-CD52 antibody in the present study is the biosimilar of Campath-1H (Hale 2001) (Drug Bank Accession number: DB00087), which was produced by a recombinant CHO cell line (TH6 cells) in-house at Shanghai Jiao Tong University (Shanghai, China) and termed mAb-TH. The TH6 cells were cultured in a Dynamics serum-free medium (Thermo Fisher, New Hampshire, USA) in both the fed-batch and perfusion processes. Six runs were performed in total (three runs for each process) using 15-L bioreactors (Applikon, Schiedam, Netherlands) at a final working volume of 12 L. An inoculum density of 0.5×10^6 to 1×10^6 cells/mL was employed in all runs, and 5 mL of sample was taken daily for cell counting and other analyses. The dissolved oxygen (DO) concentration was maintained at 40–60% air saturation. The culture pH was maintained at 6.9–7.1 by an automated monitor during the entire run, adjusted by adding either CO₂ or NaHCO₃. The temperature was maintained at 37 °C during the growth phase and was decreased to 34 °C during the stationary phase. The agitation speed was kept constant at 100 rpm. The fed-batch culture was initiated with a working volume of 9 L and was harvested when the cell viability decreased below 60%. Prior to harvesting, feed medium derived from a partial concentrate of the basal medium was added to the bioreactor every other day, and glucose was added to keep the glucose levels above 2 g/L. The perfusion process was based on a gravity settler for cell retention with an initial working volume of 12 L; perfusion was started on day 5 and eventually reached and remained at one working volume per day.

Cell density and viability

The cells were pre-stained with Trypan Blue, and the cell density and viability were measured using an automatic Countstar Cell Counter (InnoAlliance Biotech, Wilmington, DE, USA). The viability was periodically confirmed by visual examination under a microscope.

Antibody productivity parameters

Antibody (mAb-TH) titers were measured using a protein-A affinity column (Wuxi Galak Chromatography Technology, Wuxi, China) in an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA) according to the

manufacturer's instructions. An in-house produced IgG1 standard was used in the method validation.

The following productivity parameters were calculated to compare the two fermentation processes. The integral viable cell concentration (IVCC) represented the integral area from a plot of the viable cell concentration versus culture time, which was calculated as

$$\text{IVCC} = \int_0^t x \, dt \quad (1)$$

where x indicates the cell concentration and t indicates the culture period in days. The volumetric productivity (V_P , g/L/day) and specific productivity (q_P , pg/cell/day) in the fed-batch and perfusion processes were calculated from the following equations:

$$V_{P,\text{fed-batch}} = dC_P/dt \quad (2)$$

$$V_{P,\text{perfusion}} = dC_P/dt + D \times C_P \quad (3)$$

$$q_{P,\text{fed-batch}} = dC_P / \left(\int_0^t x \, dt \right) \quad (4)$$

$$q_{P,\text{perfusion}} = V_{P,\text{perfusion}} / C_X = (dC_P/dt + D \times C_P) / C_X \quad (5)$$

where C_P indicates the concentration of the accumulated antibody, D indicates the perfusion rate, and C_X indicates the cell density in the bioreactor.

Purification of humanized monoclonal antibody

To evaluate the product quality, the cell culture supernatant was purified by protein-A affinity chromatography using an AKTA Explorer (GE Healthcare Biosciences, Piscataway, NJ, USA). The eluate was adjusted to pH 7.0 and used in the following physiochemical characterization.

Peptide mapping

The antibody samples were denatured in 8 M urea solution, reduced with DTT, and alkylated with iodoacetamide (Sigma-Aldrich, St. Louis, MO, USA). Subsequently, the samples were desalted in ultrafiltration tubes with 10-kDa cut-off membranes (Millipore, Billerica, USA) and digested with trypsin (Promega, Madison, WI, USA) at 37 °C. The resulting peptide fragments were separated by reverse-phase HPLC (RP-HPLC) with a C18 column (5 μm , 250 $\times$ 4.6 mm; Waters, Milford, MA, USA) using an acetonitrile/water gradient containing 0.1% trifluoroacetic acid (Sigma-Aldrich, St. Louis, MO, USA). The absorbance was measured at 214 nm using an Agilent 1260 HPLC system equipped with a UV detector.

Antibody purity and aggregation analysis

Analytical size-exclusive chromatography HPLC (SEC-HPLC) was performed under non-denaturing conditions on a

TSK gel SuperSW mAb HR column (Tosoh, Tokyo, Japan). The isocratic elution profile was monitored at 280 nm using an Agilent 1260 HPLC system equipped with a UV detector.

Charge heterogeneity analysis

Cation exchange HPLC (CEX-HPLC) was performed to detect the charge variation of the antibody product. Antibody charge variants were separated on a 4.0 mm $\times$ 250 mm ProPac WCX-10 column (Thermo Fisher Scientific, Sunnyvale, CA, USA) using an Agilent 1260 HPLC system at a flow rate of 1.0 mL/min. Proteins were eluted at ambient temperature using a mobile phase gradient of 0–100 mM NaCl in a buffer of 10 mM MES and 10 mM HEPES at pH 8.0.

Glycosylation and sialylation analysis

In the glycosylation analysis, *N*-linked glycans were released from the antibody products by *N*-glycosidase F digestion (Roche, Switzerland) and subsequently purified by ultrafiltration with a 10-kDa cut-off. After vacuum freeze drying, the released glycan was labeled with 2-aminobenzamide (2-AB) (Sigma-Aldrich, St. Louis, MO, USA) to allow for fluorescence detection, and then purified on a DPA-6s SPE column (Sigma-Aldrich, USA). The purified glycan was separated on a TSKgel Amide-80 column (TOSOH, Japan) and quantified using an Agilent HPLC 1260 system with a fluorescence detector.

To evaluate the types and proportions of sialylated glycan in the antibody products, sialic acids were released from antibodies in 4 M acetic acid at 80 °C and labeled with DMB (Sialic Acid Fluorescence Labeling Kit, Takara, Japan). Subsequently, the labeled sialic acids were separated on a ZORBAX Eclipse XDB-C18 column (Waters, USA) and an Agilent HPLC 1260 system. The sialic acids in the sample were quantified using sialic acid standards and presented as the molar ratio of sialic acid to protein.

Complement-dependent cytotoxicity assays

Complement-dependent cytotoxicity (CDC) assays were performed by measuring the intracellular ATP levels in HuT78 cells (ATCC® Number: TIB-161™) using a Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA). Briefly, HuT78 cells were added to a 96-well flat-bottomed plate at 2×10^5 cells/well. The cells were incubated with the antibody test samples at concentrations ranging from 1 to 10 $\mu\text{g/mL}$ for 1 h at 37 °C. Subsequently, human serum was added as the source of complement. The mixture was incubated for another 2 h at 37 °C. Next, 100 μL of Cell Titer-Glo Reagent was added to each well, and the luminescence was immediately measured on a fluorescence-luminescence microplate reader (FLx 800; Biotek, USA) at

528/20 nm. Cell viability was determined by the sigmoidal dose-response curves of ATP levels. EC₅₀ (half maximal effective concentration) values were calculated using the 4-parameter statistical software GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). A human IgG (Signalway Antibody LLC, Maryland, USA) was added to validate the specificity of the anti CD52 antibody to the CD-52 antigen.

Nonclinical pharmacokinetics

Procedures, measurements, and analysis

Sixteen animals were randomly allocated to two equal groups of males and females, with each group receiving intravenous injections of fed-batch and perfusion-produced mAb-TH at 3 mg/kg three times per week for 4 weeks. Prior to the administration, sedation was induced by intramuscular (I.M.) injection of 10 mg/kg of ketamine HCl (Hengrui Medicine, Jiangsu, China). Samples for the detection of the serum mAb-TH concentration were taken 0.5 h before and 0.5, 2, 4, 8, 24, and 72 h post-dose during the first and last (12th) dose.

The concentration of mAb-TH in serum was measured using a sandwich enzyme-linked immunosorbent assay (ELISA) established based on the work by Jilani et al. (2004). In this assay, mAb-TH was captured by an in-house-prepared BSA-conjugated CD52 antigen (the amino acid sequence is QTSSPSADA-BSA), and the mAb-TH bound to CD52 antigen was detected by horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG detection antibody (Jackson Immuno Research Laboratories, West Grove, PA, USA). The assay was fully validated, and the lower limit of quantification (LLOQ) was 78.1 ng/mL. Initial PK analysis was performed using non-compartmental analysis with WinNonLin 5.2.1 (Pharsight Inc., St. Louis, MI, USA). All ELISA results were analyzed using GraphPad Prism 6 software (GraphPad Software Inc., USA).

Statistics

All analytical methods used in this study were validated prior to the measurement to ensure data accuracy and reproducibility. For the ELISA assay, the serum concentration curve was fitted to a four-parameter logistic regression. All the samples were detected in duplicates, and the result was acceptable since the % coefficients of variation (CV) was not more than 15%. Statistical analysis was performed using a two-sample equal variance (homoscedastic) *t* test with Excel (Microsoft, Redmond, Washington, USA). *P* < 0.05 was considered statistically significant.

Results

To explore the effect of different process mode on product quality, efficacy, and PK properties, a CHO cell line (TH6) expressing a humanized anti-CD52 monoclonal antibody was

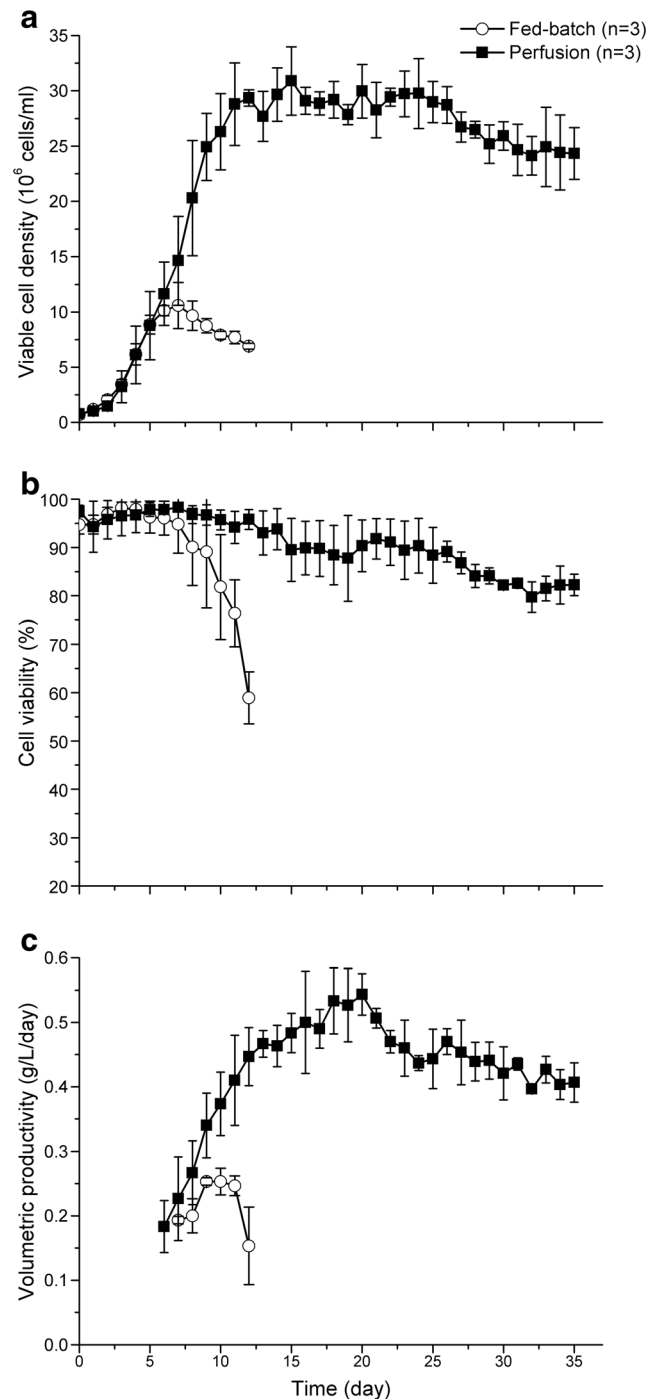


Fig. 1 Cell growth and antibody volumetric productivity of the fed-batch and perfusion cultures. A total of six runs were performed (three runs for each process). The data were presented as the mean $\pm$ standard deviation (S.D.). **a** Cell growth curves. **b** Cell viabilities. **c** Volumetric productivities

cultivated in both fed-batch and perfusion modes for three runs. The antibody produced was subsequently purified and characterized.

Cell growth and antibody productivity

The average duration of the perfusion process was 35 days, and that of the fed-batch process was 13 days. The cell density and viability and antibody volumetric productivity of the two cultures were shown in Fig. 1. The maximal viable cell density in the perfusion culture was 30×10^6 cells/mL from days 13 to 15 and that of the fed-batch process was 13×10^6 cells/mL (Fig. 1a). The cell viability decreased only slightly during the perfusion runs, whereas a significant reduction in cell viability was observed in the fed-batch culture (Fig. 1b).

The maximal volumetric productivity in the perfusion process was 0.55 g/L/day, which was 2.2-fold higher than that in the fed-batch culture (Fig. 1c). The mean specific antibody productivity (q_p) in the perfusion culture was lower than that in the fed-batch mode (16.7 vs 24.9 pg/cell/day); however, the

batch productivity of the perfusion process was higher than that of the fed-batch (perfusion vs fed-batch: batch productivity, 146.0 vs 20.5 g).

Product quality characterization

The product quality was characterized by analyzing the antibody purified from the cell culture supernatant of both the perfusion and fed-batch runs.

Tryptic peptide mappings of mAb-TH from both the perfusion and fed-batch cultures were shown in Fig. 2. Similar peptide profiles were observed for the perfusion and fed-batch cultures, indicating that the antibody primary structure was comparable between the two modes.

SEC-HPLC was used to measure the aggregation and purity of the antibody product. The proportion of aggregates and monomers was calculated by plotting the peak areas in the chromatogram, and the results were shown in Table 1. Significantly more aggregates were observed in the fed-batch culture than in the perfusion culture (2.8 ± 0.6 vs $0.9 \pm 0.4\%$). Correspondingly, a significant higher purity of

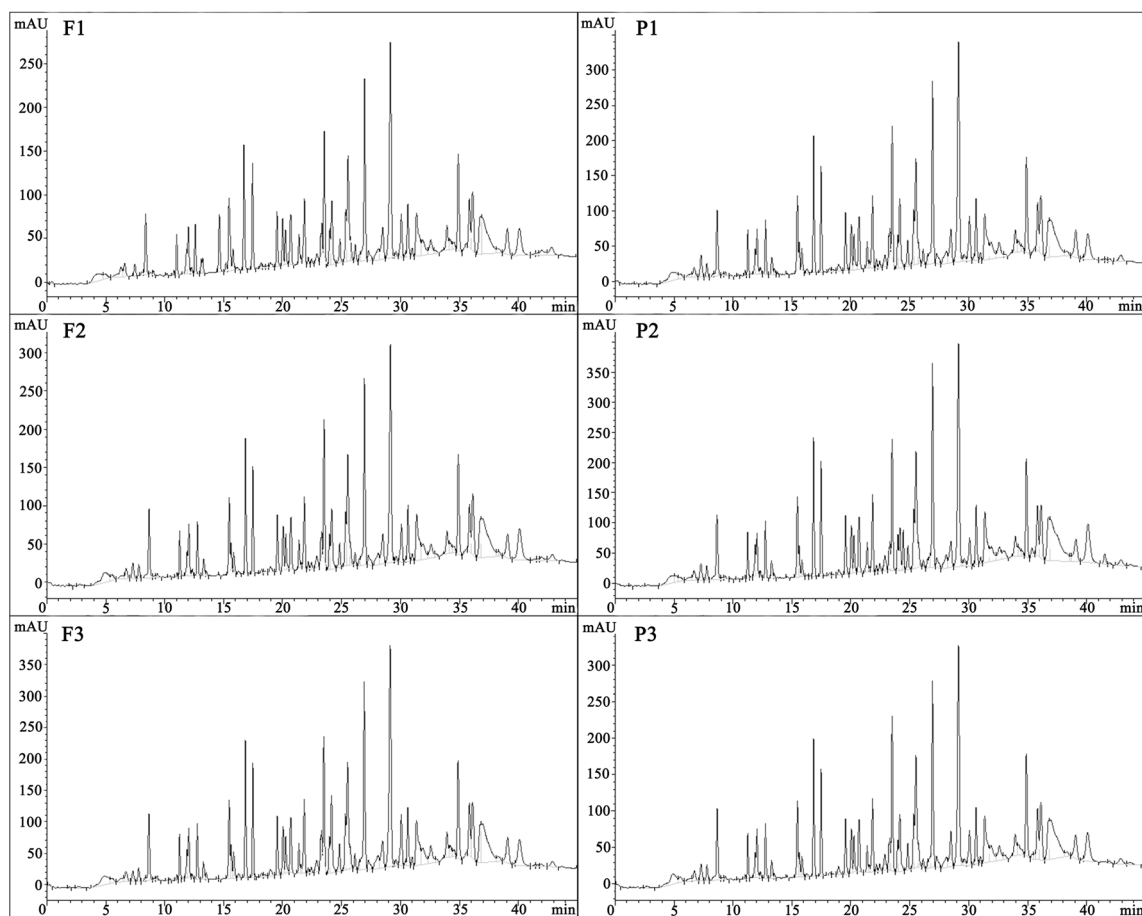


Fig. 2 Antibody produced by fed-batch and perfusion cultures showed similar tryptic peptide map profiles. F1–F3 were three runs in the fed-batch mode; P1–P3 were three runs in the perfusion mode. Similar peptide profiles were observed among the runs within and between the processes

Table 1 More aggregates were found in the fed-batch than the perfusion process

	Fed-batch ($n = 3$)	Perfusion ($n = 3$)
Monomer	$97.2 \pm 0.4\%^*$	$99.1 \pm 0.4\%$
Aggregates	$2.8 \pm 0.6\%^*$	$0.9 \pm 0.4\%$

* $P < 0.05$, compare with perfusion. Data were presented as the mean $\pm$ standard deviation (S.D.)

antibody monomer was detected in the perfusion process than in the fed-batch process (99.1 ± 0.4 vs $97.2 \pm 0.4\%$).

Charge variants from both the perfusion and fed-batch cultures were analyzed using CEX-HPLC, and the results are displayed in Fig. 3. All acid peaks, the main peak and the basic peak, were observed at similar residual times for both the perfusion culture and the fed-batch culture, although the proportions of these components were different. The percentage of the acid component from the fed-batch culture was significant higher than that from the perfusion culture (21.7 ± 1.6 vs $13.3 \pm 1.7\%$), and the proportions of the main and basic components from the fed-batch culture were lower than those of the perfusion culture (main component: 70.2 ± 1.2 vs $76.3 \pm 3.5\%$; basic components: 8.2 ± 1.2 vs $10.4 \pm 1.9\%$, respectively).

Glycosylation is generally associated with the efficacy and toxicity of a therapeutic antibody. Therefore, glycosylation patterns of an antibody drug are considered as a key parameter in drug quality evaluation. The *N*-linked glycan profiles of the antibody obtained for the perfusion and fed-batch cultures were presented in Fig. 4; the results for four major glycoforms (G0, G0F, G1F, and G2F) were plotted, as are the percentage of galactosylation (G+) and sialylation. In both processes, the *N*-glycan includes a core fucose, with 95.1 and 94.3% of the total in the fed-batch and perfusion, respectively; and terminal galactose (G0F, G1F and G2F) with varying ratios. The percentages of *N*-glycans without the core fucose were comparable between the two processes (fed-batch vs perfusion: 4.9 ± 1.2 vs $5.7 \pm 1.0\%$), while a significant higher ratio of galactose was detected in the perfusion mode (fed-batch vs perfusion: 22.3 ± 8.8 vs $38.7 \pm 4.6\%$).

Additionally, the types and ratios of sialylated glycans in the products were measured. Two major types of sialylated glycans—*N*-acetyl neuraminic acid (NANA) and *N*-glyconeuraminic acid (NGNA)—were detected from both cultures. The percentage of NANA was higher in antibody made from the perfusion culture than that from the fed-batch culture ($0.131 \pm 0.012\%$ mol/mol mAb vs $0.025 \pm 0.023\%$ mol/mol mAb, Fig. 4c). The amount of NGNA was low in both cultures and barely detectable in two runs of the fed-batch culture. Higher levels of NGNA were observed in the perfusion mode than in the fed-batch mode (0.003 mol/mol mAb vs 0.001 mol/mol mAb, respectively).

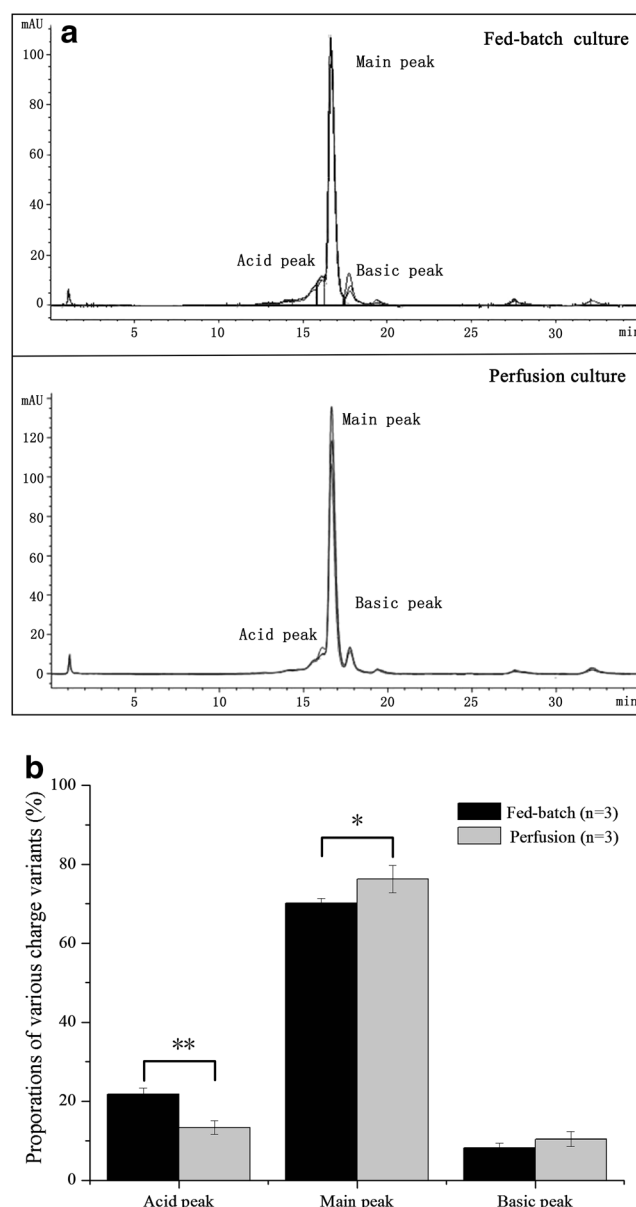
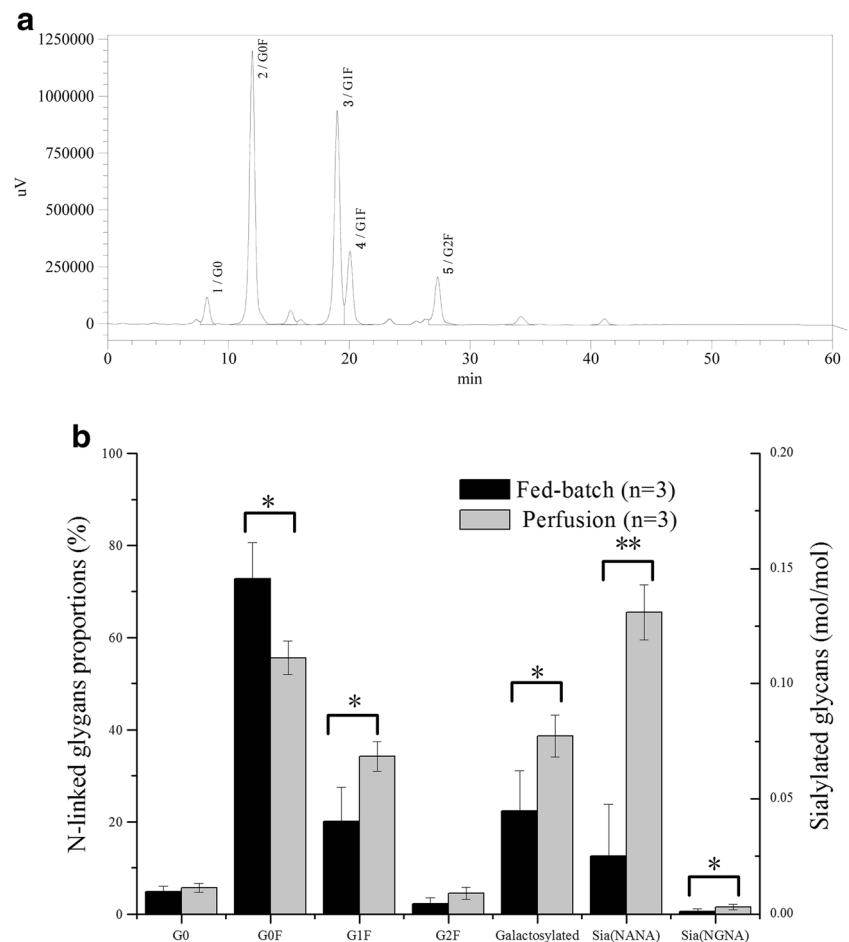


Fig. 3 Antibody produced from the perfusion process had less acid component and more main component than that from the fed-batch cultures. **a** Overlay chart of the charge distribution among the three runs. **b** Proportions of the three major peaks in three runs for either the fed-batch mode or perfusion mode. Data were presented as the mean $\pm$ standard deviation (S.D.). * $P < 0.05$, ** $P < 0.01$

The antibodies produced from the fed-batch and perfusion cultures were analyzed for CDC efficacy in a cell-based assay, and the product from the perfusion process exhibited significant higher CDC efficacy than that from the fed-batch process (Fig. 5a).

Additionally, we examined the relationship between the CDC efficacy and the terminal galactose and NANA of the product based on the values from the three runs. The results indicated that the CDC efficacy was associated

Fig. 4 Antibody produced from the perfusion process had similar fucose, more galactose and sialylated glycans than the fed-batch. **a** Chromatogram profiles of the *N*-linked glycan of antibody. **b** Percentages of *N*-glycan and sialylated glycan calculated from the three runs for each process. The data were presented as the mean $\pm$ standard deviation (S.D.). * $P < 0.05$, ** $P < 0.01$



with the levels of galactose-containing glycan (G1F + G2F) in both processes (Fig. 5b) but was not linked to the content of NANA in either the fed-batch or perfusion mode (Fig. 5c).

Nonclinical pharmacokinetics

The PK of mAb-TH produced by different cell culture modes were characterized based on multi-dose experiments in cynomolgus monkeys. During study, the animals of each group had no illness, abnormal appearance, or behavior. The mean serum concentrations of fed-batch- and perfusion-produced mAb-TH were plotted separately in Fig. 6. Cynomolgus monkeys were administered a 3-mg/kg intravenous dose of mAb-TH three times per week for 4 weeks; during the entire administration period, the mean serum mAb-TH concentration-time profiles were superimposable. The PK parameters of the first and last dose were calculated and compared (Table 2); the PK parameters C_{max} and AUC in this table were comparable between the fed-batch and perfusion groups.

Discussion

In the present study, both fed-batch and perfusion processes were performed using the same fermenters, cell line, culture media, and comparable culture conditions (e.g., temperature, pH, DO). The batch productivity of the perfusion process was much higher than that of the fed-batch, which supports that perfusion is a more efficient method than fed-batch process in the production of functional anti-CD52 monoclonal antibody. Products from different process demonstrated quality variants, including aggregates, charge heterogeneity, different glycan contents, and different CDC efficacy. Despite the differences in vitro, these variants had similar PK properties in vivo.

Aggregation is a commonly occurring during different stages of the manufacturing processes, including fermentation, purification, final formulation, and storage (Vazquez-Rey and Lang 2011). If not removed, aggregates may affect the antibody quality, efficacy, and safety. It has been speculated that the higher content of aggregate in the fed-batch process may be caused by the long exposure time of the antibody to an environment with a high temperature and a high density of cell degradation material present in the fermenter. As described

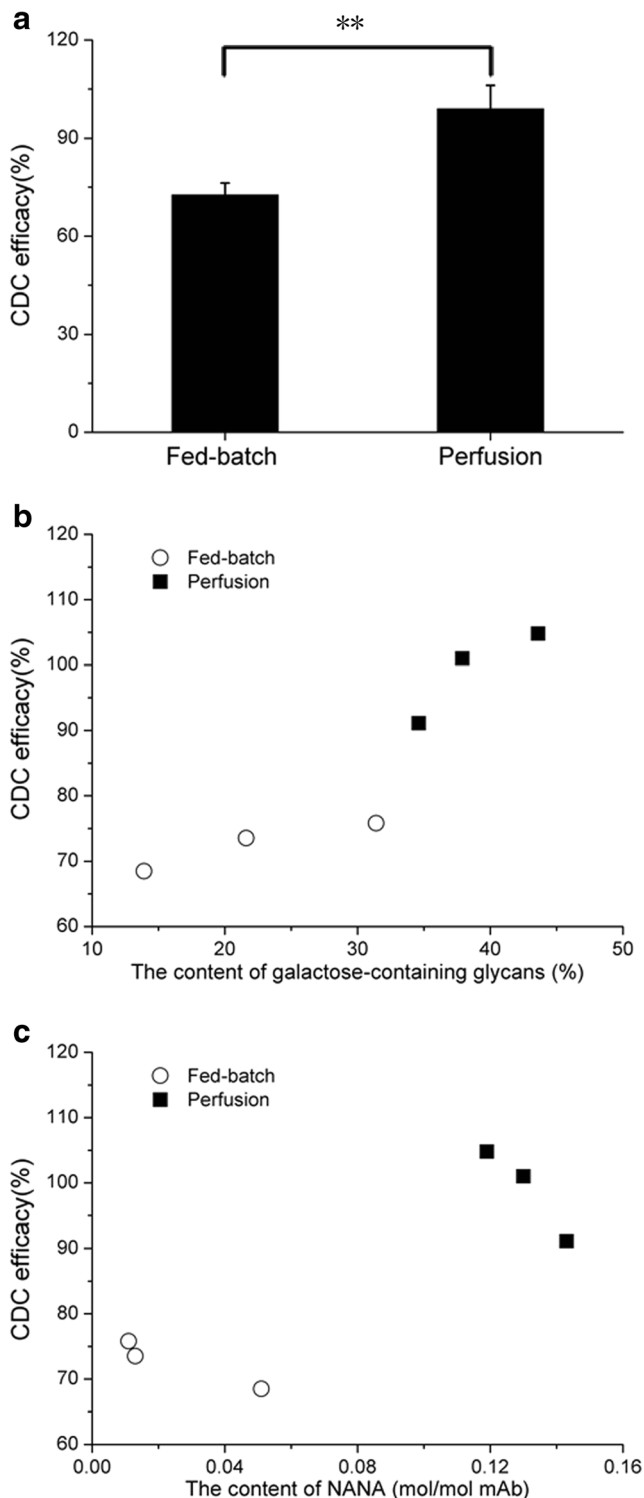


Fig. 5 Antibody produced from the perfusion process demonstrated better CDC efficacy than that from the fed-batch. **a** Comparison of the CDC efficacy of the products from fed-batch and perfusion cultures based on data from three runs. The data were presented as the mean $\pm$ standard deviation (S.D.). **b** Relationship between the CDC efficacy and levels of galactose-containing glycan from both cultures. **c** Relationship between the CDC efficacy and levels of sialylated glycan *N*-acetyl neuraminic acid (NANA) from both cultures. * $P < 0.05$, ** $P < 0.01$

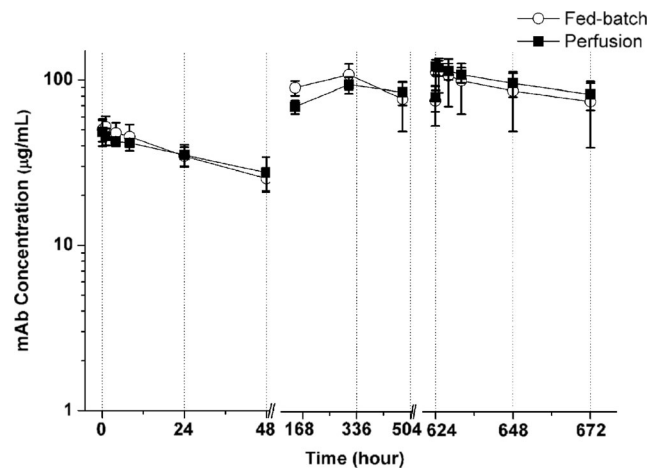


Fig. 6 mAb-TH concentrations [mean $\pm$ standard deviation (S.D.)] in the cynomolgus monkey ($n = 8$) following multi-dose intravenous administrations of 3 mg/kg mAb-TH from fed-batch and perfusion cultures, which demonstrated similar PK profile

previously, a greater amount of aggregates was observed with the extension of protein residence time (Cromwell et al. 2006). Antibody produced from the fed-batch process has a significantly longer residence time in the bioreactors than that from the perfusion culture. The residence time (τ) of perfusion was 1 day on average ($D = 1 \text{ day}^{-1}$, $\tau = 1/D$). By contrast, antibody produced via the fed-batch process in the early stage must be kept in the fermenter for nearly 10 days, during which time cell debris and high temperatures may affect the quality of the mAbs, leading to physiochemical instability or even loss of biological activity. An accelerated stability test in our previous work indicated that mAb-TH aggregates increased from 1.9 to 2.3% after exposure to an environment at 25 °C for 90 days (data not shown).

Charge variation in mAbs originates from reactions such as N-terminal pyroglutamation, C-terminal lysine clipping, oxidation, and deamidation. Ion exchange chromatography, isoelectric focusing, and capillary electrophoresis are common methods for charge profiling. Specific components can be further identified by LC-MS and LC-MS/MS (Beck et al. 2013; Johnson et al. 2007; Liu et al. 2008; Santora et al. 1999; Yang et al. 2014). In this study, a validated CEX method was utilized to obtain the charge variant profiles of mAb produced from different processes. Our results indicated that the products from the fed-batch process had a high proportion of acid components and a lower proportion of basic components. An accelerated stability study for the drug substance showed that mAb maintained under a higher temperature (data not shown) could lead to a significant increase in the acid component of the products. These studies confirm the correlation between the residual time and the acid component content of the products. Moreover, Kaneko et al. reported that mAb produced during the death phase of a recombinant Chinese

Table 2 PK parameters [mean $\pm$ standard deviation (S.D.)] from a multi-dose study in cynomolgus monkeys

	First dose		Last dose	
	C _{max} /dose (kg/L)	AUC/dose (0–48 h) (h μ g/mL)	C _{max} /dose (kg/L)	AUC/dose (624–672 h) (h μ g/mL)
Fed-batch 3.0 mg/kg	17.86 $\pm$ 2.75	533.14 $\pm$ 122.91	30.73 $\pm$ 12.49	2523.41 $\pm$ 919.46
Perfusion 3.0 mg/kg	16.09 $\pm$ 2.32	582.33 $\pm$ 82.31	33.47 $\pm$ 7.05	2725.52 $\pm$ 1684.67

C_{max} maximum serum mAb concentration, AUC area under the curve

hamster ovary (CHO) cell line had a greater acid component content (Kaneko et al. 2010).

According to our recent research, heavy-chain N-terminal pyroglutamation and light-chain C-terminal lysine clipping have been identified in the mAb-TH antibody, which may be associated with charge variants. Nevertheless, these two types of heterogeneities did not seem to have a significant impact on the antibody's safety or efficacy (Millward et al. 2008), and such variant proportion of acid and main components had no impact on the PK properties.

Antibody glycosylation can be impacted by the cell line, antibody conformation, and cell culture conditions (Wright and Morrison 1997). In this study, the different cell culture processes are primarily responsible for the variation in galactose; the mAb-TH produced from the fed-batch culture has a 42.4% decrease in the galactose level. The level of fucosylation and galactosylation of mAb-TH should be an object of focus because such glycosylation heterogeneity can significantly influence the binding affinity of the Fc fragment to receptors that mediate ADCC and CDC. Because there was no obvious difference in the fucosylation between the two processes, the ADCC assay, which is primarily influenced by fucosylation, was not performed in this study. The CDC efficacy showed a positive correlation with the galactosylation level, which has been observed previously (Boyd et al. 1995; Jefferis 2009). mAb-TH produced by the fed-batch process also had a lower sialylation level (both NANA and NGNA), likely attributed to the cell lysis removal of sialic acid from the biantennary backbone of the carbohydrates (Hossler et al. 2009). In this study, the level of sialic acid content was found to have no influence on the CDC efficacy.

The preclinical evaluation of PK is a crucial step in drug development; as a result, the change in PK profile due to a manufacturing change is also a critical assessment for a therapeutic protein. The distribution and clearance of a mAb may be affected by several aspects of the product characteristics, such as the protein sequence, structure, glycosylation, and other modifications (Han et al. 2014). In this study, the product yield from different cell culture modes resulted in comparable PK profiles, indicating that the abovementioned quality variation does not affect the distribution or clearance of mAb-TH. To supplement this, we also detected the binding affinity

of mAb-TH to FcRn (data not shown), a relevant factor contributing to the clearance of a mAb, which demonstrated that both fed-batch and perfusion produced mAb had similar affinities against FcRn, the K_D value is $2.04 \times 10^{-7} \text{ M}^{-1}$ and $1.79 \times 10^{-7} \text{ M}^{-1}$, respectively. Which indicate that the abovementioned quality variants did not alter the binding affinity of the Fc region to the FcRn receptor. Some literatures have demonstrated that mouse-originated sialic acid NGNA can cause immunogenicity in monkeys and humans (Hossler et al. 2009) and further influences the PK profile; however, we have not found the presence of NGNA to affect the PK properties. In fact, there is no consensus regarding whether the sialylation level affects the serum half-life of the antibody (Harris 1995; Scallon et al. 2007).

In conclusion, product quality variants of anti-CD52 were found in different cell culture processes, which demonstrated different physiochemical and biological activities, but comparable PK properties. Whether these observations apply to all mAb await further investigation.

Compliance with ethical standards

Ethics statement A nonclinical PK comparability study was carried out in 16 naïve cynomolgus monkeys (aged 3–5 years, weight 2–5 kg, 50% male and 50% female) provided by Hainan Jingang Laboratory Animal Co. Ltd. (Hainan, China). Cynomolgus monkeys were chosen because anti-CD52 antibody only cross-reacts with baboons and macaques, wherein only some cynomolgus monkeys have CD52 negative erythrocytes. The in vivo procedures were conducted at the National Shanghai Center for New Drug Safety Evaluation Research Center (Shanghai, China) and were approved by the Institutional Animal Care and Use Committee (IACUC) of the National Shanghai Center for New Drug Safety Evaluation Research Center (Permit Number: IACUC-2015-M-012).

The housing and experimental procedures were carried out strictly according to the United States National Research Council's *Guide for the Care and Use of Laboratory Animals*. Animals were maintained in separate cages of $1000 \times 750 \times 930 \text{ mm}^3$ size during daytime and paired housing was applied overnight. The temperature was maintained at 16–24 °C, the relative humidity was 40–70%, air changes occurred at least eight times per hour, and the illumination time was 7 AM to 7 PM. The animals were fed monkey chow (Ke Ao Xie Li, Beijing, China) and fresh fruits and vegetables. The cages were composed of stainless steel with a lookout, perches, and a stainless steel mirror. To alleviate animal suffering, procedures were performed under sedation with anesthesia by intramuscular (I.M.) injection of ketamine HCl (Hengrui Medicine, Jiangsu, China), animals were trained prior to the venous sampling with positive

reinforcement, and fruits were offered after sampling. No animal that participated in this study was sacrificed.

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

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