Process Biochemistry 48 (2013) 1572–1580

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Effects of the dilution rate on cell cycle distribution and PEI-mediated transient gene expression by CHO cells in continuous culture

Li Wei-wei[a,1], Liu Shao-jiao[a,1], Chen Yan-tian[a,*], Zheng Chen[a,b,1,2], Qi Nian-min[a,*]

[a] Cell Culture and Bioprocess Engineering Lab, School of Pharmacy, Shanghai Jiao Tong University, Dongchuan Road 800, Minhang District, Shanghai 200240, China
[b] Shanghai Taiyin Biotechnology Co. Ltd., Room 305, Cailun Road 781, Zhangjiang Hi-tech Park, Shanghai 201203, China

ARTICLE INFO

Article history:
Received 20 May 2013
Received in revised form 30 June 2013
Accepted 2 July 2013
Available online 10 July 2013

Keywords:
Cell cycle distribution
Transient gene expression
Transient transfection
Recombinant protein
Cell cycle synchronization
Continuous culture

ABSTRACT

In this study, a continuous culture system was applied to mammalian cells on large scale, and polyethyleneimine (PEI) mediated transient gene expression (TGE). PEI MAX 40,000 was chosen as a superior reagent from three types of PEI. The cell cycle distribution of cells in batch and continuous cultures was determined, in which the effects of cell cycle distribution on transfection efficiency, post-transfection proliferation and recombinant prothrombin expression were evaluated. Compared with cells from end-log and plateau phase in batch culture, cells from mid-log phase possessed a larger fraction of S and G2/M phase cells and a smaller fraction of G1 phase cells. In the continuous culture, the fraction of cells in the S and G2/M phases increased and the fraction of cells in the G1/G0 phase decreased with increasing dilution rates. Cells from the continuous culture run at highest dilution rate had excellent proliferation, transfection efficiency and protein expression. These results were confirmed by transfecting cells synchronized to different phases. The G2/M arrested cells exhibited a nearly 10-fold increase in recombinant human prothrombin production relative to that of non-dividing cells. The use of continuous culture for large scale transfection demonstrated a better cell physiological state for TGE process.

1. Introduction

Genetically engineered, recombinant proteins (r-proteins) are of great commercial and scientific interest for their investigative, preclinical and pharmaceutical uses. An increasing demand for r-proteins up to the milligram or gram scale emphasizes the need for a breakthrough in process development. In contrast with the different r-protein expression systems that have already been established, such as *Escherichia coli*, yeast, insect cell and stable mammalian cell lines [1–5], transient gene expression (TGE) in mammalian cells can offer a less time- and labor-consuming solution supply r-proteins with correct folding and posttranslational processing at early stages of development [6–8]. During the last decade, the TGE process has been studied and improved by numerous scientific groups and pharmaceutical companies to overcome the obstacles to TGE application, that is, the incredibly low titers and low specific productivity relative to stable cell lines [1,9,10].

In these studies, cationic polymer polyethyleneimine (PEI)-mediated transfection was considered to be more suitable at large scale because it exhibits several properties such as high efficiency in a broad range of different cell lines, low cytotoxicity, simplicity and cost-effectiveness [11–13]. Human embryonic kidney 293 (HEK293) cells and Chinese hamster ovary (CHO) cells are the most commonly used host cell lines. Both cells are easily adapted to serum-free suspension culture [14], which ensures their application to large-scale TGE in bioreactors. HEK-293 cells and its genetic variants (such as 293-EBNA1 and 293-T) cell lines are the most widely used because of their high transfection efficiency, autonomous episome replication and high titer yield. CHO cells (including CHO-K1 and CHO-S), which were initially used to produce stable cell lines for r-protein expression, were also used because it was reported that the post-translational modification of proteins from HEK293 and CHO cells are different [15,16]. Thus, it would be ideal to use the same host cells lines to produce r-proteins by TGE and stable cell lines because they exhibited similar N-glycan processing [17]. With the exception of the cell source and transfer reagent, these studies also involve the optimization of other key aspects which determine the r-protein yield such as (but not limited to) the expression vector, transfection parameters, medium and post-transfection culture mode [18,19].

In addition to the aspects mentioned above, it was commonly agreed that cells in a good physiological state are crucial for

* Corresponding authors. Tel.: +86 021 34204745; fax: +86 021 34204745.
E-mail addresses: ytchen@sjtu.edu.cn (Y.-t. Chen), ccbe_sjtu@outlook.com (N.-m. Qi).
[1] Tel.: +86 021 34204745; fax: +86 021 34204745.
[2] Tel.: +86 021 34206671.

1359-5113/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.procbio.2013.07.002

transient transfection and those from the mid-log growth phase are optimal for TGE. These cells, which possess the highest specific growth rate, were believed to provide a higher transfection efficiency and stronger tolerance to toxicity generated by high concentrations of PEI and other additive reagents, in which the reagent is used to enhance transfection efficiency and protein expression. Cell growth was regulated by the cell cycle, which consists of three distinct phases, namely, the gap 1 phase (G1 phase), the DNA synthesizing phase (S phase) and the gap 2/mitotic phase (G2/M phase). There is an additional resting phase (G0) into which cells will enter when the conditions are unfavorable for growth, and then they become non-cycling cells. Cells in different phases of the cell cycle exhibit different properties with respect to their size, metabolic activity and consumption rates. In an asynchronous culture, the apparent values of these properties are actually the average values of the whole population. Consequently, the cell cycle distribution in an asynchronous culture will determine the properties of TGE cells. Thus, the effects of cell cycle distribution on TGE need to be clarified.

In the literature, the traditional way to obtain cells in the mid-log phase is to maintain cells in batch culture and frequently passage them every 2–3 days to prevent cells from "aging", and then resuspend them one day prior to transfection [20]. However, there are several shortcomings to this "standard" protocol. For instance, this protocol is not well-suited for large scale TGE because it increases the process complexity, the workload and the chance of contamination. Moreover, batch-to-batch variations in transfection efficiency and protein expression may occur. This phenomenon is mainly caused by cell variability from batch cultures. Consequently, the batch-to-batch consistency and process robustness are addressed as critical issues for cases when no r-proteins generated by TGE were currently approved by FDA. Continuous culture systems can give a more stable, constant and sustained environment for cell growth. As a consequence, the batch-to-batch variation can also be minimized. Thus, a study on their potential use in large scale transfection is necessary.

In our study, the application of continuous culture systems for large scale transient transfection is presented. Three PEI reagent and CHO cells were used to investigate the optimal transfection conditions for prothrombin production. The cell cycle distribution of cells in batch culture and continuous culture were measured, and their effects on transient transfection were evaluated to establish the optimal continuous culture parameters. Cell synchronization was also used in the transient production of prothrombin to explain the difference in transient transfection between cells in different phases of cell cycle.

2. Materials and methods

2.1. Plasmid DNA

pGMAX-FII vector coding for recombinant human prothrombin (also known as human coagulation Factor II, or rhFII) was constructed based on vector pGMAX, which was kindly provided by Dr. David Lee of the School of Pharmacy, Shanghai Jiao Tong University. This vector contains the IRES element followed by the enhanced green fluorescent protein (eGFP) element. The human elongation factor 1 alpha (EF1α) promoter is used to increase transient gene expression. The plasmid was amplified in *E. coli* DH5a cells (Invitrogen, USA) and then purified using a commercially available kit (Tiangen, China). Purified plasmids were analyzed by agarose gel electrophoresis and the concentration of plasmid DNA was determined at 260 nm with a spectrophotometer. Only the plasmid, whose A_{260}/A_{280} ratio is between 1.8 and 2.0, was used for transfection.

2.2. Cell line and medium

CHO-S cells were obtained from Invitrogen and adapted to serum-free suspension culture in Ex-cell CD-CHO medium (Sigma, USA) supplemented with 8 mM glutamine. CHO-S cultures were maintained at viable cell densities between 3×10^5 and 3×10^6 viable cells/mL in 125 mL Erlenmeyer shaker flasks with vented caps (Corning, USA) at 120 rpm on an orbital shaker in an incubator (Thermo, USA) set at standard conditions (37 °C, 5% CO_2). The adapted cells were stored in liquid nitrogen to be used as a working cell bank. Viable cell densities and cell viabilities were determined by trypan blue exclusion. Glucose and lactate concentrations were determined with commercial kit.

2.3. Batch culture and continuous culture

2.3.1. Batch culture

CHO-S cells in mid-log phase were harvested and resuspended at a viable cell density of 1×10^5 cells/mL in fresh culture medium in 125 mL shaker flasks (20 mL working volume) placed in an incubator (120 rpm, 37 °C, 5% CO_2), respectively. Cells were cultured for 11 days without any nutrient addition or medium exchange. Cells were sampled every day, cell and the cell density, viability, cell cycle distribution, glucose and lactate concentrations were determined.

2.3.2. Continuous culture

The experiments were carried out in a continuous culture system. In brief, this system consists of a 500 mL spinner flask (20 mL working volume) (BD Falcon, USA), pump tube (MasterFlex, USA), fresh medium and waste reservoir and two peristaltic pumps (LongerPump, China) set at the same rate. The whole system was placed in an incubator at standard condition of 120 rpm, 37 °C, and 5% CO_2. CHO-S cells in mid-log phase were harvested and seeded at a density of 3×10^5 cells/mL in fresh culture medium. Two days after the beginning of the culture, the dilution was started and the dilution rates were adjusted (if necessary) according to an optimized strategy for cell growth. The dilution rate was kept constant to allow the cultures to reach a steady state. Cells were sampled every day for cell density, viability, cell cycle distribution, and glucose and lactate concentrations.

2.4. Theory

CHO cells were cultured in continuous culture systems at different dilution rates. Because there is no accumulation of viable cells at steady state, the cell growth and cycling time can be calculated by Eqs. (1) and (2),

$$\mu = \frac{D}{v} \tag{1}$$

$$t_C = \frac{\ln 2}{\mu} \tag{2}$$

where D is the dilution rate, v is the viability (%), μ is specific growth rate and t_C is the cycling time.

The frequency of cells located at time t from the beginning of the G1 phase is proportional to $2^{1-t/t_C}$ [21]. Given the fractions (%) of cells in each phase of the cell cycle, the residence times (hours) of cells in certain phases are given by Eqs. (3)–(5) [22]:

$$t_{G1} = \frac{-\ln(1 - 0.5 \times f_{G1})}{\mu} \tag{3}$$

$$t_S = \frac{-\ln(e^{-\mu \times t_{G1}} - 0.5 \times f_S)}{\mu} \tag{4}$$

$$t_{G2/M} = t_C - t_{G1} - t_S \tag{5}$$

where f_{G1}, f_S and $f_{G2/M}$ is the fractions of cells in each phase of the cell cycle, t_{G1}, t_S and $t_{G2/M}$ is the residence times of cells in each phase.

Knowing the fraction of G0 phase cells, the cycling time can be calibrated according to Eq. (6), followed by the calibration of residence times.

$$t_C = \frac{\ln 2}{\mu} \times (1 - f_{G0}) \tag{6}$$

2.5. Transfection protocol

Three different PEI molecules, namely, -PEI MAX 40,000 (PEI MAX), linear 25 kDa PEI (25 kDa l-PEI) and linear 2.5 kDa PEI (2.5 kDa l-PEI) (Polysciences, USA) were used as transfection reagents. A stock solution (2 mg/mL) of PEI was prepared in a buffer solution (150 mM NaCl, 10 mM HEPES, pH 7.9), filter sterilized and stored at room temperature. Before transfection, the cells were counted and resuspended in RPMI 1640 (Gibco, USA). Purified plasmid DNA and PEI stock solution were separately added to the equivalent of 5% of the final culture volume in buffer solution (150 mM NaCl, 10 mM HEPES, pH 7.1) and kept at room temperature for 5 min. PEI was then added to the DNA solution, and the mixture was rapidly vortex for 5 s. The PEI/DNA complexes were incubated at room temperature. This prepared cocktail was then added to the cell culture. When the transfection was finished, a complete exchange back to the culture medium was performed. For small-scale transfection, experiments were performed in non-tissue culture treated 12-well plates (Corning, USA) to ensure the cells remained in suspension. Cells taken from the mid-log growth phase were seeded into fresh medium at 1×10^6 cells/0.9 mL/well and then transfected with pGMAX-FII vector. For large scale transfection, experiments were performed in 125-mL shaker flasks (20 mL working volume) or 500 mL spinner