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100-liter transient transfection

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Abstract

This is the first report of two successful 100 l scale transient transfections in a standard stirred bioreactor. More than half a gram of a monoclonal antibody (IgG) were produced in less than 10 days using a technology called large-scale transient gene expression (LS-TGE). Suspension adapted HEK 293 EBNA SF cells were transfected within a 150 l (nominal) bioreactor by a modified calcium phosphate co-precipitation method with more than 75 mg of plasmid DNA per run. A mixture of three different plasmids, one encoding for the heavy chain of a human recombinant immunoglobulin, the other for the corresponding light chain and a third one for the green fluorescent protein (GFP, 2–4% of DNA in transfection cocktail) were co-transfected. The GFP vector was chosen to monitor transfection efficiency. Expression of GFP could be registered as early as 20 h after DNA addition, using fluorescence microscopy. We demonstrate that transient transfection can be done at the 100 l scale, thus providing a new tool to produce hundreds of milligrams or even gram amounts of recombinant protein. A key advantage of LS-TGE resides in its speed. In the presented cases, the entire production process for the synthesis of half a gram of a recombinant antibody, including DNA preparation and necessary expansion of cells prior to transfection, was executed in less than a month. Having an established transfection/expression process allows to run production campaigns for any given protein, within one facility, with one single host cell line and therefore only one single seed train. Without any need to create and maintain stable cell lines, expression of new r-proteins is not only faster and more economical but also more flexible.

Introduction

In the development of medically active proteins, the time to market is the most important cost factor. If a specific r-protein is lost to another competitor, all investments in the project are gone. Every year of delay before going to market adds additional costs in the range of several tens to hundreds of millions of dollars on the bill for drug development and validation (Aunins, 2001). Tools providing research and/or clinical grade material more rapidly are most welcome. The quantities of recombinant proteins (r-protein) must be sufficient to allow large animal testing, development of downstream processing, stability testing and further characterization of the respective proteins.

We present the scale-up of the classical calcium phosphate co-precipitation method (CaPi) (Graham and Eb, 1973; Jordan, 2000; Jordan et al., 1996, 1998; Meissner et al., 2001) to scales beyond one hundred liters of culture volume. Transient transfection at large scale would be able to provide hundreds of milligrams or even gram amounts of a desired recombinant protein within days.

The r-protein expressed in two successful runs was a human anti-RhD-IgG (Miescher et al., 2000).

Materials and methods

Cell culture

4×10^7 HEK 293 EBNA SF (Girard, 2001) cells were thawed and resuspended directly into 50 ml of chemically defined Pro293s-CDM (BioWhittaker Inc., Walkersville, MD) medium in a spinner flask. The cells were gradually expanded to larger spinners (Bellco Glass Inc., Vineyard, NY). The seeding densities ranged from 2×10^5 cell ml^{-1} to 5×10^5 cells ml^{-1}. To improve maximal cell density the cultures were supplemented with 2% FCS (JRH Biosciences, Lexana, KS). The cells were cultivated in a 5% CO_2 atmosphere at 37 °C. Spinner flasks with culture volumes of more than 1 l were cultivated in a 37 °C warm room. All spinners were stirred at 80 rpm. 13 l spinners were actively aerated using a simple tube, a vent filter and a peristaltic pump. The cells were passaged as soon as lactate levels exceeded 1.5 mg ml^{-1}. Glucose and lactate levels were measured with a multi-parameter analyzer (NOVA Biomedical, Waltham, MA).

DNA

The DNA was prepared using commercial purification kits (Nucleobond AX, Machery-Nagel, Düren, Germany). The IgG was expressed using a vector for the light chain (LH1) and one for the heavy chain (LH2) in a ratio of 3 to 7. As a marker for transfection efficiency, plasmid pEGFP-N1 (Clontech, Palo Alto, CA) was co-transfected. Table 1 shows the DNA amounts and ratios used for the full-scale transfections in the 150 l bioreactor. Overall 75 mg of DNA were transfected per run.

Transfection

Transfection buffers
Solution A: 250 mM calcium solution ($CaCl_2 \cdot 2H_2O$, Fluka Chemie AG, Buchs Switzerland) in ddH_2O. Solution B: a 50 mM HEPES and 140 mM NaCl solution prepared with ddH_2O, pH 7.05. 1.4 mM $PO_4{}^{3-}$ from a 300 mM stock solution ($Na_2HPO_4 \cdot 2H_2O$, all Fluka) were added. Solutions A and B were filtered at 0. 22 μm and autoclaved.

Cell preparation
After cell expansion in large spinners, the cells were centrifuged for 10′ at 330 g in individual 1000 ml beakers at 5 °C. The supernatant was decanted and the cells were resuspended in 2 l of FEME Plus (DMEM/F12 (Gibco, Scotland) fortified with 2.5 mg l^{-1} insulin, 2.5 mg l^{-1} transferrin, 2.5 mM L-glutamine (5 mM total concentration), 1.85 g l^{-1} glucose (5 g l^{-1} total concentration), 10 mM HEPES (all Sigma, St. Louis, MO), 0.1 mM diethanolamine, 0.1 mM L-proline (both E. Merck, Darmstadt Germany), and 2% FCS) and recentrifuged at 160 g for 5′. After this washing step, the cells were seeded in 30 l FEME Plus into the reactor (150 l total volume, BioEngineering) and incubated for 2 h at a stirrer speed of 200 rpm. For the exact seeding densities and other culture parameters see Table 2.

Transfection
The DNA was added to 1500 ml of calcium solution (A) under a laminar flow hood and gently mixed. 1500 ml of phosphate buffer (B) were added to form the calcium-phosphate-DNA complex (CaPi). The solutions were well mixed, the flask closed and connected to the reactor using sterile couplings. After exactly 1 min, the transfection mixture, containing the transfection particles, was transferred into the reactor using compressed air for the 1st run and a high capacity peristaltic pump for the second run. The transfection cocktail was incubated on the cells for 6 h.

In order to dissolve the CaPi transfection complex, the pH in the reactor was shifted from 7.5 to 7.2, and 30 l of FEME Plus were added. Half an hour later, 600 ml of an antibiotic, antimycotic solution were added together with 0.1% (w/v) of Pluronic F-68 (both Sigma).

Production and harvest

Samples were taken twice daily. The cell density was measured indirectly by packed cell volume (PCV). The viability was assessed with Trypan blue staining. pH and dO_2 were measured offline (NOVA) and corrected on the reactor's transmitters. Glucose and lactate levels were measured. The IgG content was analyzed by ELISA (Jordan et al., 1999; Miescher et al., 2000). To release the GFP, the cells from the reactor were lysed by repeated freezing and thawing cycles.

Antifoam C, diluted 1:10 and autoclaved, was added as needed to eliminate foam. Stirrer speed was gradually increased up to 250 rpm to control the cell aggregate size. The pH was regulated at 7.1 and controlled with NaOH 1 M and CO_2. The dO_2 was held constant at 35%. During the first run, the headspace was continuously flushed with fresh air. The air sat-

Table 1. DNA amounts and ratios used for large-scale transfection. LH1 expresses the light chain and LH2 the heavy chain of the antibody. The pEGFP-N1 is used as a control for transfection efficiency

Plasmid	1st Run		2nd Run	
	DNA (mg)	Percentage	DNA (mg)	Percentage
LH1	22.1	29.5%	22.1	29.0%
LH2	51.4	68.5%	51.3	67.1%
pEGFP-N1	1.5	2.0%	3.0	3.9%

Table 2. Summary of relevant transfection and cultivation parameters of the two large-scale transfection runs. The first run was a simple batch culture. The second run was an extended batch culture with sodium butyrate addition to increase the r-protein production

Parameter	1st Run	2nd Run
Cells	HEK 293 EBNA SF	
Cell density	350 000 cells ml^{-1}	585 000 cells ml^{-1}
Expansion medium	Pro293s-CDM 2% FCS	
Transfection medium	FEME Plus 2% FCS	
Transfection method	CaPi	
Antibiotics	Present during production phase	
Vectors	Heavy chain light chain 7/3 2% pEGFP-N1	Heavy chain/light chain 7/3 4% pEGFP-N1
Total DNA quantity	75.0 mg	76.3 mg
Volumes	At transfection: 33 l After transfection: 67 l At harvest: 67 l	At transfection: 33 l After transfection: 66 l At harvest: 110 l
Culture duration	10 days	
Antifoam	N/A	Yes
Agitation	150–250 rpm	
Aeration	Surface aeration only	Bubble aeration
Extended batch	No	Yes, 40 l at day 3
Butyrate addition	No	Yes

uration was held constant by sparging air into the culture.

The first reactor was conducted as simple batch cultivation. On day 5, the cells were fed with 200 g glucose. The supernatant was harvested on day 10 after transfection. The second reactor was operated as extended batch culture with butyrate induction. Three days after transfection, 40 l of FEME Plus were added. 12 h later 100 g of glucose were added. On day 7, 0.2 mM of sodium butyrate were added. After 9 days, 50 l of supernatant were harvested. On day 10 the remaining cell suspension was harvested.

In Table 2, all the parameters concerning the two large-scale transfection runs are summarized.

Results

Cell expansion

To generate the cell mass needed for the large-scale transfection, cells were expanded in spinner flasks. HEK 293 EBNA SF suspension cells grew routinely to densities higher than 2×10^6 cell ml^{-1} and reached maximal cell concentrations of 2.5×10^6 cells ml^{-1}. Even at the highest cell densities, the cultures mainly grew as single cell suspension.

For larger spinners with more than 3 l of culture volume, it was necessary to force air into the culture to achieve cell densities comparable to those seen in small-scale spinner cultures. For cell densities around

2×10^6 cell ml^{-1}, forced aeration was necessary to assure relative air saturation higher than 30% and to strip CO_2 out of the spinner to reduce the pH drop in the culture. No obvious disadvantage was observed on whether to expand the cells with a dilution or with a centrifugation step.

Transfection and production

Addition and mixing times are most critical in the formation of transfection effective transfection complexes (Jordan et al., 1996), see the section Discussion. The calcium solution and phosphate buffer were mixed within 11 (10, values for 2nd run in brackets) seconds; the transfer into the reactor started 50 (40) seconds after the addition of the phosphate. The transfer into the reactor was completed after 30 (35) sec. The cells were transfected at pH 7.53 (7.51). The addition of the CaPi complex shifted the pH to 7.35 (7.35).

In FEME Plus medium, cells started to aggregate. To minimize aggregation, the initial stirrer speed of 150 rpm for cell seeding was increased to 200 rpm during the conditioning to shear aggregates. During transfection and incubation of Calcium-Phosphate-DNA complexes with cells, the stirring intensity was reduced to 150 rpm to limit dissociation of cells and CaPi particles.

During the incubation time, the pH decreased gradually from 7.35 (7.35) to 7.20 (7.25). After 6 h, the transfection suspension was diluted with 30 l (40 l) of pre-warmed medium and the pH was shifted to 7.1 to further enhance the CaPi complex dissolution.

To limit the size of cellular aggregates the stirrer speed was increased 24 h after transfection to 200 rpm and on day 2 to 250 rpm.

24 h post-transfection, the cells visibly expressed GFP in both runs. For the first run with only 2% of GFP encoding plasmid, 50% of the cells expressed the fluorescent protein 72 h after transfection. For the second run with 3.9% of the transfected DNA encoding for GFP, 60% of the cells expressed protein, as shown in Figure 1.

After transfection, the cells grew as aggregates of several thousands cells. Most of the individual cells that detached from the aggregates were non-viable. r-Proteins were mostly expressed within the aggregates (Figure 1). Figure 2 shows that the production of the intracellular GFP stops after 100 h, while secreted IgG production still continues.

Volume specific IgG production in the reactor reached 4 mg l^{-1} after 10 days for the first run and 5 mg l^{-1} for the second extended batch run, during which butyrate was added. The overall production titers steadily increased for both runs over the whole production period to 260 mg in the first and more than 500 mg in the second run.

Even without medium feeding for the first run, it was possible to keep the viability within the aggregates above 90% for as long as 8 days. On day 9, the viability fell to 50% and reached a low 10% at the end. With feeding additional medium, in the second run, the viability looked very similar to the first run. Viability in the aggregates did not drop below 95% in the first 8 days after transfection. By the end of the run, the viability gradually dropped to 60%. pH and dO_2 were constant during the whole production run. Growth pattern, glucose consumption and lactate production during the whole culture were comparable to smaller scale runs.

Discussion

Cell expansion

The use of very large spinners, 13 l, for the expansion culture with a filling volume up to 8.5 l. was only possible with forced aeration. This ensures an efficient stripping of the CO_2 out of the culture medium and also provides enough oxygen for the culture. The possibility to dilute the culture during cell expansion allows a fed-batch strategy in the spinners, starting at a small volume of around 2 l and periodically increasing the culture volume with fresh medium. This reduced manipulations and the risk of contaminations. Sterile couplings on the culture vessels helped further to reduce the manipulations as the spinners could stay in the warm room for sampling and media addition.

Transfection and production

Batch wise centrifugation is the most commonly used cell separation technique. The main disadvantage of this technology for large volume application is its non-continuous mode of operation. Repeated batch centrifugation has the advantage of being an easy to use technology. The conditions during centrifugation are gentle. The cell separation with batch centrifugation is clearly limited to smaller volumes, up to 18 l, corresponding to three centrifugation runs and a washing run, and a total process time of 40 to 60 min. For large volumes of culture, the cells should be harvested

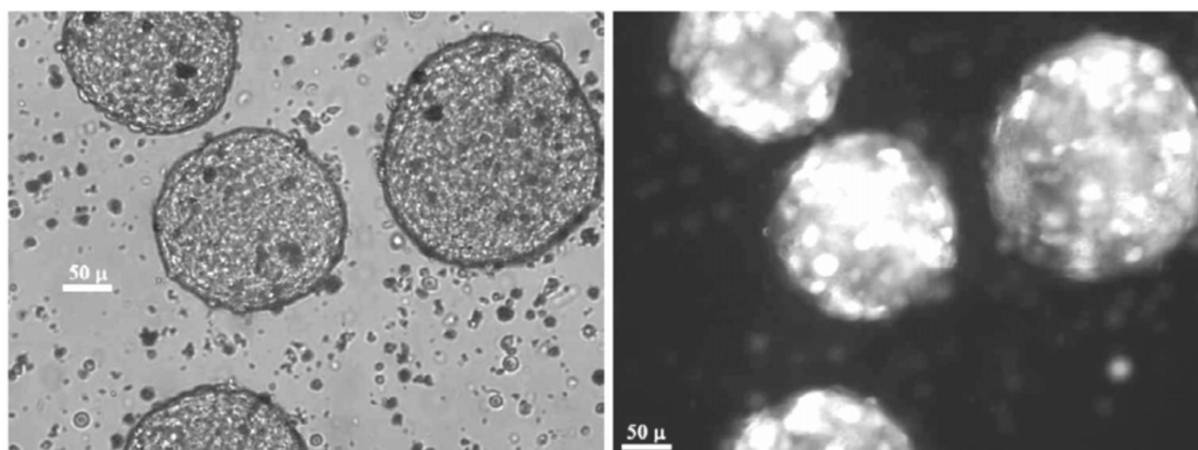

Figure 1. Cells 3 days after transfection in the reactor, with visible light on the left and UV illumination on the right. 60% of the cells express GFP (right) despite the fact that only 3.9% of the DNA encoded for the fluorescent protein. The cells grow as aggregates of several thousand cells. In order to avoid nutrient and oxygen diffusion limitations, the stirrer speed was increased gradually to keep a constant aggregate size (Pictures from the second run).

differently. For large-scale transfections, the centrifugation was used to keep the process as simple as possible.

Due to the lower viscosity and therefore higher sedimentation velocities of the cells, the cultures should be centrifuged at higher temperatures, e.g. 37 °C. To allow cells to survive in a close packed pellet they should be stored on ice. The cold storage, even for short periods of time, reduces the cell's metabolism avoiding cell damage and an extended lag phase after centrifugations due to depletion and lack of oxygen in the pellet.

Washing the cells prior to transfection, after large-scale centrifugation, is crucial in order to completely remove the expansion medium. The same agents that hinder cell-to-cell contact and aggregation also limit cell-CaPi-complex interaction and therefore the transfection. Traces of this medium inhibit transfection to a considerable extent (data not shown).

Liquid handling and transfection

The use of pressurized air to transfer the CaPi complex into the reactor has the advantages of a faster transfer and a lower dead volume compared to a liquid handling system with a peristaltic pump. Fast addition is needed to guarantee a defined complex formation time, and to ensure the formation of an effective transfection complexes of reproducible size. The time window for suspension transfection is around 30 to 120 sec for an optimal transfection (data not shown). In addition, pressurized vessels are also better protected against contaminations as nothing can enter the sterile environment due to the pressure difference. Alternatively, high capacity peristaltic pumps can be used.

The transfection complex must be dissolved, otherwise its toxic effects (Jordan et al., 1996) will override the beneficial effect of a longer incubation time that would allow the cells to take up transfection particles over an extended period. Adding extra medium and lowering the pH can readily achieve this goal (Meissner et al., 1999).

Aggregation

Due to the higher Ca^{2+} concentration (Peshwa et al., 1993) cells tend to aggregate during transfection and thereafter (see Figure 1 as example). Cell aggregation may reduce the potential contact surface between cells and transfection complexes, therefore reducing the effectiveness of the transfection. Large aggregates are also undesirable during the production phase due to the diffusion limitations for oxygen and nutrient supply within the aggregates. This can lead to cell death within larger cell clusters. Increasing the stirring speed is an efficient tool to control aggregate size and therefore to avoid cell death inside larger cell aggregates. Increasing the stirrer speed and the power input creates a broader shear stress distribution and a higher peak stress causing larger cell clusters to fragment.

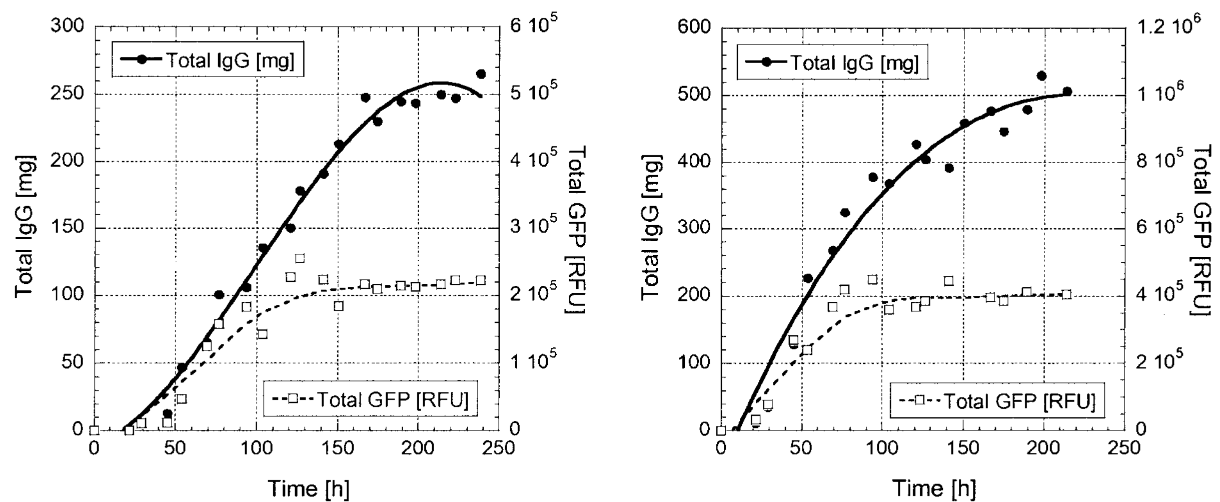

Figure 2. The left hand side shows the total amount of r-protein produced in the first run with a total volume of 110 l. The right hand side shows the total protein production of the second run with a final volume of 65 l at harvest. The bold lines indicate the overall tendency of the expression (polynomial models). The total IgG amount of antibody in the reactor reached 260 mg for the first run and more than half a gram for the second. The overall production of GFP compared to the production of IgG shows clearly that after about 100 h there is no further production of GFP, but IgG production continues.

Production

Co-transfecting a fluorescent marker protein allows for rapid tracking of transfection efficiency by simply using a florescence microscope. The presence of e.g. GFP offers the possibility to directly assess the transfection efficiency, the proportion of r-protein expressing cells and all the cells.

The difference in the protein expression pattern between the GFP and the IgG could be an indication that the plasmids encoding for IgG were episomally replicated, since they contain the oriP origin of replication (Yates et al., 1985). The pEGFP-N1 on the other hand, without oriP and no large T-antigen expressed in the cells, is expected to get lost. The graphs in Figure 2 give support for this hypothesis.

Sodium butyrate has been reported to increase production of stable and transiently expressed r-proteins (Gorman et al., 1983; Kruh, 1982; Parham et al., 1998). Whether there is an overall beneficial effect of the butyrate addition for the production of run 2 cannot be clearly stated.

Conclusion

Transient gene expression can be scaled-up to 100 l. The process is still in an early stage of development. The principle has been proven but there is still a long way to go. Future efforts must focus on process refinement and on the development of tools and the conception of equipment to reduce and to simplify manipulations e.g. liquid handling, cell separation. New equipment should lower the risks of contamination. This would also lead to a process without antibiotics, easing up downstream processing.

The FCS dependence of the large-scale transfection is still a major drawback of the process with its impact on downstream processing and purification. The substitution of FCS with albumin or chemically defined additives seems to be a possible solution. The best solution would be to develop chemically defined media suitable for transfection.

Another critical point is the complete removal of the growth medium, BioWhittaker Pro293s-CDM, which is not suitable for transfection. For the presented runs, batch wise centrifugation was used to recover the cells from the expansion culture. This separation method is clearly limiting further scale-up. A promising solution for larger volumes is continuous centrifugation. It is also imaginable to develop a new medium that would allow the expansion of the cells and the transfection without a change of medium. A supplementation strategy for the medium prior to and after transfection could be the key to such a development.

The actual process is robust at scales up to 100 l. It is therefore a fast alternative to r-protein production in stable cell lines. The major advantage lies in

the speed of the r-protein expression. For standard stable r-protein production, even the first step, creation of a stable cell line takes several months. With transient expression, several production runs, including plasmid production and method optimization, can be performed in a much shorter time.

Having an established transfection/expression process allows running production campaigns for many different r-protein with one single host cell line. It is therefore possible to produce many different proteins with only a single seed train. Already today, this can further reduce the fixed costs for the production of research and diagnostic grade r-proteins. In addition to these advantages, there is no need to create or maintain stable cell lines. Expression of r-proteins is not only faster and more economical but also more flexible with large-scale transient gene expression (LS-TGE).

In the presented examples, the entire production process for the synthesis of up to half a gram of a recombinant antibody, including necessary expansion of cells prior to transfection, was executed in less than a month.

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