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Calcium phosphate transfection optimization for serum-free suspension culture

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Abstract

Aim of this study was to identify optimal conditions for suspension transfection in the absence of serum. Transfection parameters for suspension culture can be very different to ones in adherent cells. Most transfection protocols have been developed and optimized for adherent culture.

Using green fluorescent protein (GFP) as reporter, FCS was eliminated from the transfection process by altering critical parameters and by substituting serum with albumin. Using standard phosphate and calcium concentrations for transfection in the absence of serum resulted in titers of only 1% of those observed in the presence of serum. A reduction of the calcium concentration from 250 mM to 100 mM, yielded a 25-fold increase in the expression of the recombinant protein compared to the serum-free standard conditions. Altering the phosphate concentration, 1.4 mM in the transfection buffer, did not improve the protein expression. Interestingly, reduction of DNA quantity by half to a concentration of 0.5 μg per milliliter of culture volume resulted in a two-fold increase of protein production. Addition of albumin to serum-free medium protected the cells against the toxicity of the calcium phosphate transfection particles (CaPi) yielding higher protein expression. All the experiments were executed in a shaken multi-well system, allowing high multiplicity parameter screening to speed up optimizations. The culture system is inexpensive, simple and efficient, minimizing costs for labor and consumables.

Introduction

Demand for recombinant proteins (r-proteins) is steadily increasing. Bacteria, yeasts, plants and animals, as well as cell and tissue cultures of plant, insect and mammalian cells are commonly used as expression systems for r-proteins. Usually mammalian cells are used to achieve suitably glycosilated r-proteins that are of therapeutical value.

Microgram amounts of r-proteins for research purposes can be produced fast by transient gene expression (TGE) (Paborsky et al., 1990). For larger scale protein expression TGE was not suitable so far, since adherent cell cultures have only limited scalability. More recently, suspension TGE protocols have been established and transfections up to the 20-liter scale have been reported (Wurm and Bernard, 1999).

Adherent cells can be transfected at high efficiency using the calcium phosphate (CaPi) technique (Graham and Eb, 1973; Strain and Wyllie, 1984; Wilson et al., 1995; Jordan et al., 1996; Seelos, 1997; Wilson and Smith, 1997). The method is also applicable to suspension cells (Chu and Sharp, 1981; Yen et al., 1988; Song and Lahiri, 1995; Jordan et al., 1998; Meissner et al., 1999). The current protocols are only successful in the presence of fetal calf serum (FCS). However, serum can interfere with downstream processing and complicate protein recovery.

The objective of this work was to eliminate the FCS from the transfection process, to express r-proteins in a chemically defined environment. In addition, serum-free operations ease-up downstream processing. To achieve this aim, the three most critical parameters for CaPi method were optimized for

Figure 1. Shaken multi-well system. 12-well microtiter plates on a rotational shaker can readily simulate stirred tank bioreactor conditions. pH, temperature and humidity are controlled by a CO_2-incubator. All transfection experiments were carried out in this system.

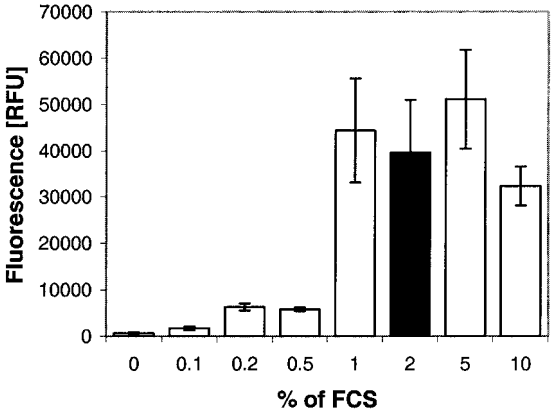

Figure 2. Influence of FCS concentration on the expression of GFP. For the standard transfection conditions, 1.4 mM Pi, 250 mM Ca^{2+} and 2.5 mg DNA/ml of transfection volume, the minimal FCS contents is 1%. Higher concentrations do not significantly increase the protein expression. Standard FCS supplementation for transfection shown in black.

serum-free suspension transfection: the concentrations of calcium, phosphate and DNA used.

Materials and methods

HEK 293 cells were maintained in serum-free suspension in a chemically defined media. (Pro293s-CDM, BioWhittaker, USA) in spinner flasks (Bellco Glass Inc., USA or Integra Biosciences, Switzerland).

The plasmid pEGFP-N1 (Clontech Laboratories Inc., USA) encoding for green fluorescent protein (GFP) was used as reporter. The DNA was purified on anion exchange columns (Nucleobond® AX, Machery-Nagel GmbH and Co. KG, Germany). Plasmids were stored at 1 μg μl^{-1} in Tris-EDTA-buffer, pH 7.4 (Tris; BHD Laboratory Supplies, England / EDTA; Fluka Chemie AG, Switzerland).

Transfection experiments were done in a small-scale (2 ml) microtiter plate system. A rotational shaker (KS250 basic, IKA, Germany) was installed in a CO_2-incubator (Heraeus BB16, Kendro Laboratory Products, Germany). 12-well microtiter plates (TPP, Switzerland) were stacked on the insulated agitator plate allowing several hundred 'bioreactor' experiments to be run in parallel, see Figure 1. With a shaking diameter of 20 mm, a rotation speed of 200 rpm was found to be optimal.

Standard DMEM/F12 supplemented with 2.5 mg l^{-1} insulin, 2.5 mg l^{-1} transferrin, 2.5 mM L-glutamine, 1.85 g l^{-1} glucose (all Sigma Chemical CO, USA), 10 mM HEPES (BHD), 0.1 mM diethanolamine, 0.1 mM L-proline (all E. Merck, Germany) was used for the transfections. This medium is referred to as transfection and production medium (TPM). 2 hours prior to transfection 600,000 cells in 1 milliliter of TPM, serum-free or supplemented with 2% FCS, were seeded into each well. Transfection complexes were prepared in small reaction vessels by mixing gently DNA with 50 μl of autoclaved Ca^{2+} solution. 50 μl of an autoclaved phosphate (Pi) solution containing 140 mM NaCl (Merck) and buffered at pH 7.05 with 50 mM HEPES (Sigma), were added per well to form the CaPi-complex. After exactly 60 seconds, the transfection mixture was transferred to the well. The transfection cocktail was incubated on the cells for 4 hours. 1 ml of pre-warmed TPM was then added to the culture to dissolve the transfection complex.

Calcium ($CaCl_2 \cdot 2H_2O$) concentration of the transfection buffer was varied between 60 and 250 mM, phosphate ($Na_2HPO_4 \cdot 2H_2O$, all from Fluka) concentration between 0.1 and 2.0 mM. DNA amounts per well ranged between 0.6 and 5.9 μg. A full screening with five different concentrations for each parameter was done in triplicate for serum-free conditions. For the substitution experiments ovalbumin (Sigma) was used. Standard transfections for expression comparison with FCS (SeraTech, Germany) were carried out

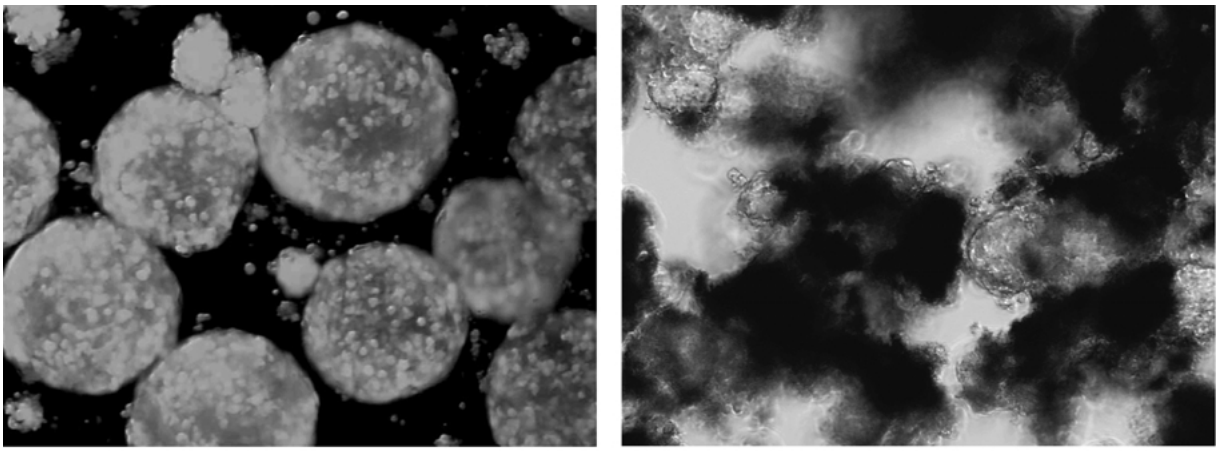

Figure 3. The cells on the left are transfected in the presence of 1% FCS with pEGFP-N1. The cells grow as aggregates of several thousand cells; the picture shows only cells fluorescing green, expressing GFP. Transfection efficiency was higher than 80%. On the right the cells were transfected using standard transfection parameters (see text) in the absence of FCS, no fluorescence was detectable. The picture shows the culture in visible light, most cells are dead.

with 2.5 μg DNA/well, 250 mM Ca^{2+}, 1.4 mM Pi and 2% FCS.

Results and discussion

FCS dependence

The CaPi transfection technique, described for large-scale transfection in bioreactors for suspension culture, requires the presence of 2% FCS (Jordan et al., 1998; Meissner et al., 1999). Figure 2 shows that the expression of GFP strongly depends on the presence of serum. For standard transfection conditions, a minimal contents of 1% FCS is needed for efficient GPF expression. Higher serum concentrations do not significantly improve the protein expression.

Visual inspections of the cells transfected under serum-free conditions showed a dramatic decrease of viability within 45 minutes after addition of the CaPi transfection complex. This was not observed under serum containing conditions (Figure 3). It appears that FCS has a protective role against negative effects associated with CaPi transfection. This is supported by the fact, that addition of serum in the medium for the dilution step, after CaPi incubation with the cells, can be omitted without loss in expression. On the other hand, addition of high quantities of FCS after the execution of a serum-free transfection has no beneficial effects (data not shown). All further optimizations were done in the absence of serum, except for control transfections for comparison.

In the presence of FCS, elevated calcium concentrations during and after transfection induce formation of moderate size aggregates. However, cells remain highly viable and continue to grow as aggregates. Under serum-free conditions, a few hours after transfection aggregation is much more pronounced, large clusters of cell debris and chromosomal DNA dominate the culture, see Figure 3. Most cells have died and very little protein expression is observed.

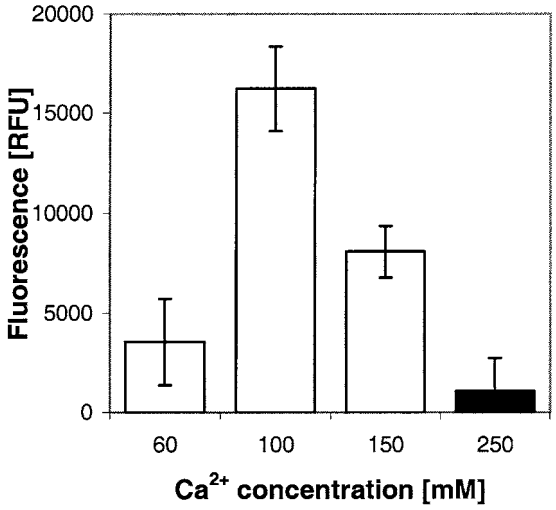

Figure 4. For serum-free transfection lowering the Ca^{2+} concentration form 250 mM to 100 mM leads to an increase of the GFP expression by a factor 5 to 10 compared to standard conditions (in black). Transfection parameters were 1.0 μg DNA/well (1 ml transfection volume) and 1.4 mM Pi.

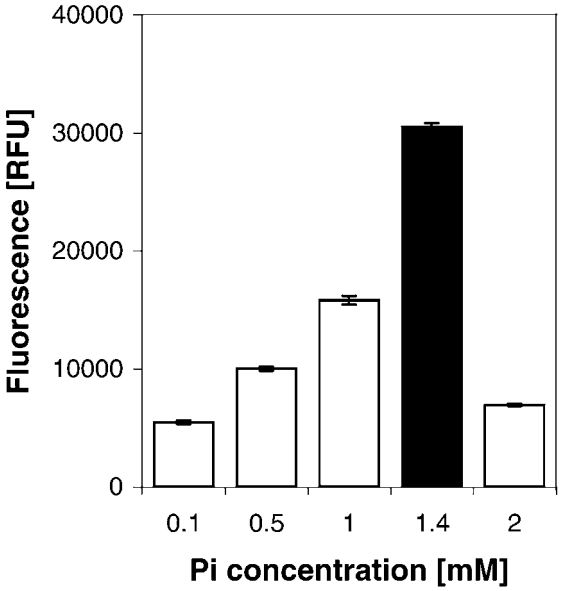

Figure 5. The standard Pi concentration (in black) is optimal for transfection and the expression of the GFP in a serum-free culture. The Pi concentration influences the nucleation of the CaPi complex. Transfection parameters were 1.2 μg DNA/well (1 ml) and 100 mM Ca^{2+}.

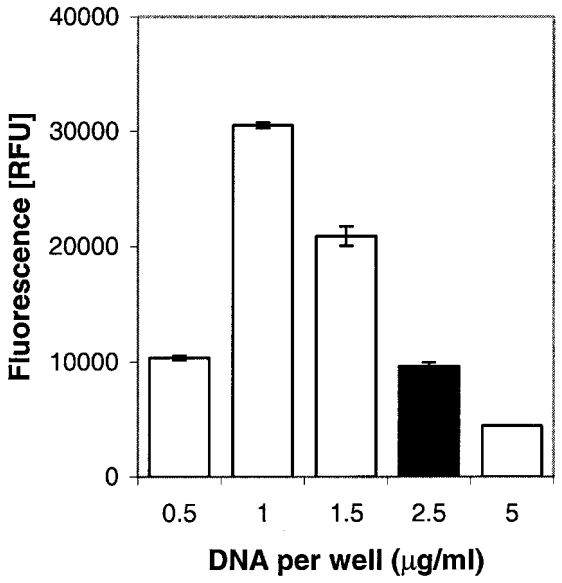

Figure 6. Optimization of the DNA amount used for serum-free transfection. The standard amount of 2.5 μg DNA per milliliter of transfection volume was reduced by half and the overall GFP expression was increased two-fold. Transfection parameters were 1.4 mM Pi and 100 mM Ca^{2+}.

Calcium concentration

Earlier studies (Jordan et al., 1996) have shown that calcium ion concentration influences the charge of the transfection complex and its size. The size of the CaPi complexes is important for an efficient DNA-uptake. The smaller the complexes are, the easier the uptake. But calcium also induces cell aggregation in suspension culture and can have toxic effects. Lowering the Ca^{2+} concentration of the transfection solution for serum-free transfection to 100 mM increased expression by a factor of 5 to 10, compared to the standard serum-free transfection, as shown in Figure 4.

Phosphate concentration

The Pi concentration also influences the CaPi size and charge (Jordan et al., 1996). Altering this parameter did not improve protein expression. The Pi concentration has its primary influence on the nucleation and crystal growth during complex formation. The standard 1.4 mM Pi was found to be optimal, as shown in Figure 5.

DNA amount

The DNA amount influences the formation of the transfection complex. During complex formation, the DNA is first condensed by the calcium ions. This complex is further precipitated with the phosphate to form a highly efficient transfection particle, usually called transfection complex.

Providing sufficient quantities of highly pure DNA can be a limiting factor for transfections in bioreactors. This motivated us to explore reduction of the DNA quantity used. With optimized solutions, 100 mM Ca^{2+} and 1.4 mM for Pi, we could reduce the DNA amount, with a benefit on gene expression. The standard amount of 2.5 g DNA per milliliter of transfection volume was reduced by half and the overall GFP expression was increased two-fold. Figure 6 shows the results obtained.

Albumin substitution of FCS

Since albumin is one of the major components in FCS, with 50 g per liter, we explored it as a substitute whole serum. As a single defined component, albumin has, for production purposes, obvious advantages over the undefined mixture of components in serum. Ideally, for concerns on bio-safety, r-protein (human) albumin would be better than any animal or human placenta derived fractions. However, recombinant albumin is limited in availability and high in price. Figure 7 compares albumin supplemented with a serum containing

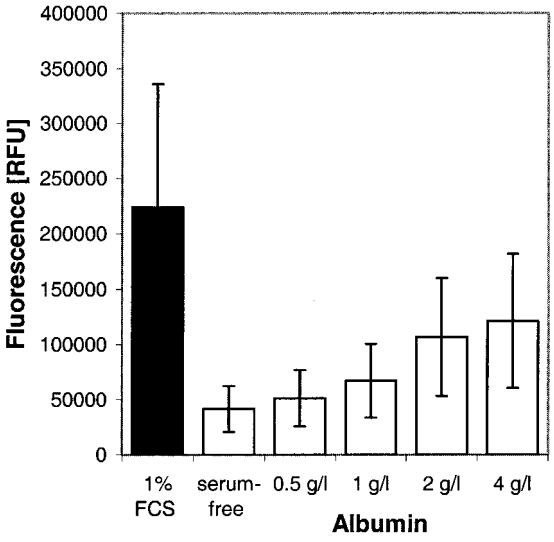

Figure 7. Albumin addition to the serum-free media increases the protein expression. Half of the expression levels achieved with the serum containing protocol can be reached by substituting the serum-free medium with albumin.

transfection medium. While we could not achieve a level of protein expression similar to that seen in transfections in serum containing media, the results show that supplementation of a serum free medium with a defined component of serum can provide good transfection efficiencies and significant recombinant protein expression.

Conclusions

The small-scale system for suspension cell culture is a highly valuable tool for the optimization of transfections in suspension. It allows screening of numerous parameters in parallel experiments. For scale-up, the system offers a good correlation to larger systems as spinners or even bioreactors, as shown earlier (Girard et al., 2001).

For the classical CaPi transfection, omitting FCS from the procedure yields different results, whether the cells grow adherently or in suspension culture. Comparable expression levels were obtained for the adherent system with or without FCS (data not shown). For suspension culture, the presence of FCS plays a crucial role. Transfection without FCS using standard parameters results in a protein expression 100 times lower than with serum. However, decreasing the calcium concentration and the DNA amount, an expression level 50 times higher could be reached in chemically defined serum-free conditions, supplemented with albumin. This represents 50% of the serum containing protocol yield. The substitution of FCS by large amounts of albumin could further enhance the expression levels. Albumin substitution of FCS might not be the best solution for the transfection in a chemically defined culture. High protein concentrations can also hinder efficient downstream processing. On the other hand availability of recombinant albumin, best suited for a defined and save process, is limited in availability. Other components, with a high potential to protect the cells from the toxic effects of the CaPi transfection complex, such as lipids or blends of lipids have not been tested yet. Most of the cells die early during the CaPi complexes incubation, this decrease in viability correlates with the rapid aggregation observed in those cases. The influence of the FCS is poorly understood. A better understanding of the effects of serum would be helpful to choose more effective substitutes for FCS. Serum might protect the cells during transfection by coating the cells and/or the transfection complex, by supplying protective factors or maybe also by forming chelates with the calcium ions reducing their concentration within the culture.

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