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Calcium-phosphate mediated DNA transfer into HEK-293 cells in suspension: control of physicochemical parameters allows transfection in stirred media

Transfection and protein expression in mammalian cells

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Abstract

This is the first report of transient transfection of suspended cells with purified plasmid DNA in bioreactors or spinner flasks. DNA/calcium phosphate complexes were pumped or injected directly into stirred cultures of the immortalized human embryo kidney cell line 293 (HEK-293) which had been adapted to growth in suspension. We identified culture conditions suitable for this approach and modified the protocol for the generation of precipitate complexes, based on our earlier work. In order to stabilize the 'DNA-vehicle'-complex in the culture medium, we identified pH ranges and ion-concentrations which prevent dissolution or aggregation of the precipitate particles. Such conditions maintained suspended fine particles in spinners or bioreactors for up to 6 hr. During that period, cells and precipitate complexes interacted sufficiently to allow DNA transfer and subsequent expression of recombinant protein. In a simple 5 day batch process, with a starting density of 0.3×10^6 cells mL^{-1}, about 0.5 mg L^{-1} of a recombinant tissue plasminogen activator variant was observed.

Introduction

Calcium phosphate mediated transfection has, since its introduction more than 20 yr ago (Graham et al., 1974), continued to be the most frequently used technique for plasmid DNA transfer to mammalian cells *in vitro*. This popularity in cell biological and biomedical research is based on its simplicity. Briefly, molecules of the recombinant plasmid DNA are co-precipitated in a super-saturated calcium phosphate solution. The plasmid DNA molecules become incorporated in emerging calcium phosphate precipitates. This reaction mixture is then added to cell culture dishes. The precipitate settles on the surface of the host cell membrane and is taken up by phagocytosis while cells are exposed to the precipitate (Loyter et al., 1982). During this period of time the pH of the culture medium is critical for the transfection efficiency (Chen et al., 1987; Gorman et al., 1990; Graham et al., 1983). It is suspected that the pH would mainly affect precipitate stability and therefore influence transfection rate. The presence of an appropriate precipitate has been shown to be a key factor for successful transfection of adherent cells (Chen et al., 1987; Graham et al., 1974).

Our previously reported improvements of calcium phosphate protocols for transient and stable transfection of human embryo kidney cell line HEK-293 and DHFR deficient Chinese hamster ovary cells (Jordan et al., 1996) provided motivation to move forward to the step from adherent to suspension cell cultures. The major concern was that for transfection of cells in stirred cultures hydrodynamic forces superimpose the gravity force. We expected that mixing, provided by the impeller, would mediate 'collisions' between cells and precipitates and that such brief interactions would be sufficient to have the particles adhere to the cells.

The environment in bioreactors and spinners is, however, clearly different from culture dishes. Media stirring might result in a different requirement for calcium and phosphate concentrations in order to maintain the precipitate as a stable entity with potential drawbacks for transfection rate and cell vitality. Also any manipulation of the medium in a reactor is more difficult due to the larger fluid volumes. The goal was therefore to develop a novel protocol adapted to the needs and restrictions of cell cultures maintained in stirred reactors.

Materials and methods

Plasmid isolation

Bacterial cultures (E. coli strain DH5α) containing plasmids of interest were grown in LB medium and DNA was isolated using standard alkaline lysis protocols. All plasmids were purified by two consecutive equilibrium CsCl gradient ultracentrifugations (Sambrook et al., 1989).

Transfection in plates

HEK-293 cells (Paborsky et al., 1990; Pear et al., 1993) from exponential growth phase were seeded at 2×10^5 cells per well into 12-well plates 20 hr prior to the transfection (1 mL medium/well). Fresh medium was provided 1 hour before the precipitate was added. The precipitation mixture was generated as described earlier (Jordan et al., 1996). 100 μL (25–160 μL for the experiment described in Figure 2) of the precipitation mixture was added to each well 1 min after mixing. The plate was incubated at 37 $^\circ$ for 4 hr. Medium was subsequently exchanged and the cells incubated at 37 °C. β-gal positive cells were counted microscopically. X-gal staining was applied 24 hr after transfection (Sanes et al., 1986). TNK-tPA (Paoni et al., 1993) concentration in the cell culture supernatant was analyzed by ELISA.

Binding of DNA

300 μL of the precipitation mixture was centrifuged for 30 s at 16 000 g in a 1.5 mL Eppendorf tube. The co-precipitation of DNA was determined by OD measurement at 260 and 320 nm using a 250 μL aliquot of this supernatant.

Transfection in spinners

Cells were maintained in 250 mL spinner flasks in a DMEM/F12 based medium containing 2% fetal calf serum by sub-cultivation once or twice per week. The same medium also was used for the transfection experiments. In order to eliminate any variations within an experiment due to the cell source, cells were either taken from a single spinner flask or were pooled. Exponentially growing cells were harvested by centrifugation and resuspended in pre-warmed medium at 0.6×10^6 cells mL^{-1}. 100 mL of this cell suspension was transferred to a 250 mL spinner flask (Bellco Microcarrier Spinner Flask) and incubated at 37 °C for 1–2 hr before the transfection was started. At this point, the pH of the medium was 7.4–7.6. The calcium concentration was first increased by adding 3 mL of 250 mM $CaCl_2$. Then 1 mL of 2xCa/DNA-solution (two-fold concentrated Ca/DNA solution containing 200 μL of 2.5 M $CaCl_2$ and 100 μg of plasmid DNA diluted in 1 mM Tris HCl, 0.1 mM EDTA, pH 7.6) was mixed quickly with 1 mL of 2xHEPES-solution (140 mM NaCl, 1.3 mM Na_2HPO_4, 50 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), pH = 7.05 at 23 °C) and added to the cell suspension one minute later. After another 4 hr, 100 mL of fresh medium was added and the cells were incubated for 21–6 days.

Transfection in bioreactors

The pH of the bioreactor was added 30 min before transfection (experiment 6A). The calcium concentration was elevated to 10 mM (7.5, 10 and 14.5 mM in 6B) by addition of 2.5 m calcium chloride stock solution, 1 min before adding the precipitation mixture. For each 2 L-bioreactor, containing a volume of 1.2 L of cell suspension (standard: 0.3×10^6 cells mL^{-1}), 25 mL of precipitation mixture was prepared. Precipitation was initiated by transferring quickly 12.5 mL of 2xCa/DNA into 12.5 mL of 2xHEPES (see above) in a sterile 200 mL flask. Within 60 to 90 sec, this precipitate was drawn into a 60 mL syringe and injected into the reactor via an aseptic membrane. 4 hr later, 0.8 L of fresh medium was added and the pH was adjusted to 7.2.

Results and discussion

Solubility of calcium in cell culture medium

In preliminary studies we found that addition of a certain amount of calcium chloride to cell culture medium leads to a very fine, but clearly visible precipitate. This was assumed to be a form of calcium phosphate which was formed from phosphate present in the medium.

The effect of calcium on precipitate formation was investigated systematically and various pH values between 6.8 and 7.6 were tested. Different amounts of calcium were added to 12-well plates containing serum-free DMEM/F12 medium buffered with 30 mM HEPES. After overnight incubation at 37 °C in 5% CO_2-atmosphere, precipitate formation was assessed using phase contrast microscopy with 400X magnification. In Figure 1, the lowest calcium concentration resulting in precipitation, is plotted as a function of the medium pH. The data demonstrate that increasing the pH in the medium reduces the solubility and lower concentrations of calcium lead to a visible precipitate.

For transfections the DNA-calcium phosphate co-precipitate is first formed in the absence of medium at calcium values much higher than needed to guarantee an over-saturated solution: 125 mM calcium is suggested for a pH of 7.05 (Graham et al., 1974). Transferring the precipitation mixture to the cell culture results in a 10-fold dilution, setting a final calcium concentration of about 12.5 mM in the culture medium. Even if a significant fraction of calcium would be bound by cells, cellular proteins (in cell conditioned medium) and notably by serum proteins (Guyton, 1991), this concentration of calcium prevents the dissolution of precipitate in DMEM/F12 medium at a pH of 7.2 or higher.

Transfection efficiency as a function of the medium pH

To find out whether the influence of the pH on the transfection efficiency can be explained by an effect on the solubility of calcium phosphate, a series of experiments was performed using media adjusted to four different pH values (between 6.8 and 7.9). For each pH five different calcium concentrations, ranging from 4 to 20 mM, were tested. In order to vary the calcium concentrations, different volumes of the precipitation mixture were added to the cells (note that more calcium resulted in more DNA on a per cell basis).

The transfection experiments were, as a first step, done in 12-well plates with pre-attached HEK-293 cells. Immediately before the transfer of the precipitation mixture, the standard medium was replaced to shift the pH. The transfection was performed with a β-gal expression vector which allowed assessment of the efficiency of DNA transfer by counting β-gal positive cells using light microscopy. At the lowest pH of 6.8, no positive cells could be detected at a calcium concentration of 8 mM or less, as shown in Figure 2 (representative for transfections repeated with the same or with other cell lines). Only a few positive cells appeared at a concentration of 12.5 mM calcium. At the same pH when using 20 mM calcium, however, the transfection efficiency was high and the number of positive cells was comparable to transfections done at higher pH. With increasing pH, the minimum concentration of calcium required to detect significant numbers of positive cells decreased and was about 6 mM at a pH of 7.9. Although it appears that pH 7.4 gives the best results, cells can in fact be transfected at any physiological pH if calcium concentrations are adjusted accordingly.

Transfection efficiency as a function of phosphate concentration in the medium

Another medium constituent, which is expected to affect the solubility of precipitated DNA, is phosphate. For one pH value, pH 7.1, six medium phosphate concentrations were studied, ranging between 0.25 and 4 mM, combined with three different calcium concentrations (more calcium resulted in more DNA on a per cell basis). At the lowest concentration of phosphate, the precipitate dissolved after it had been added to the cell culture and no precipitate could be detected after 4 hr, when assessed by phase contrast microscopy. No β-gal positive cells were found at this phosphate concentration (Figure 3). The standard concentration of 1 mM of phosphate resulted in the highest transfection efficiency with 18 and 12 mM calcium, whereas 6 mM calcium only resulted in DNA transfer at the highest phosphate concentrations (2 and 4 mM phosphate). At 2 or 4 mM phosphate, the precipitate in the plates was more coarse in nature and it appeared that a larger quantity of precipitate covered the cells, however, the transfection efficiency was reduced. 6 mM calcium combined with 2 mM phosphate resulted in a precipitate very similar in nature to the 12.5 mM/1 mM calcium/phosphate case, but gave a lower transfection efficiency.

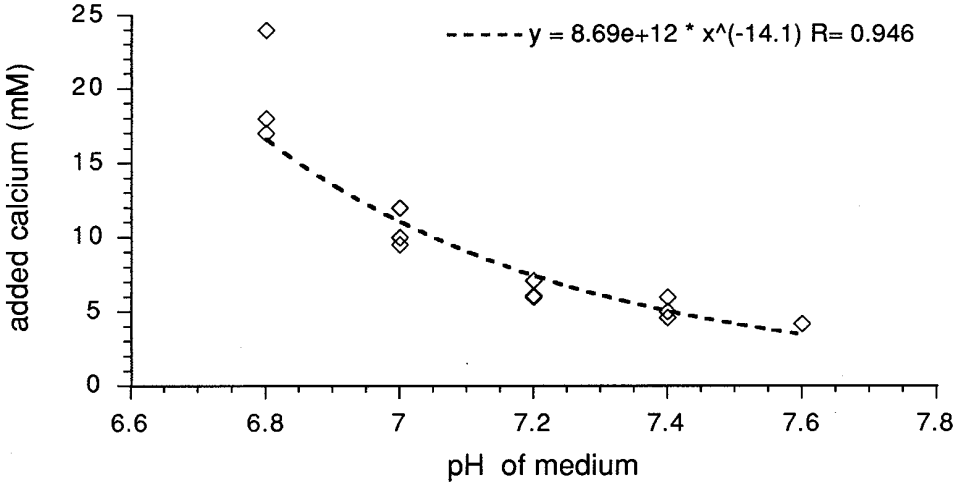

Figure 1. Solubility of calcium in cell culture medium at 37 °C as a function of pH of the culture medium. Different amounts of calcium were added to serum-free DMEM/F12 medium. Individual wells were examined for precipitate particles the following day. The lowest concentration of calcium resulting in a precipitate, as visualized by phase contrast microscopy at 400X magnification, was plotted.

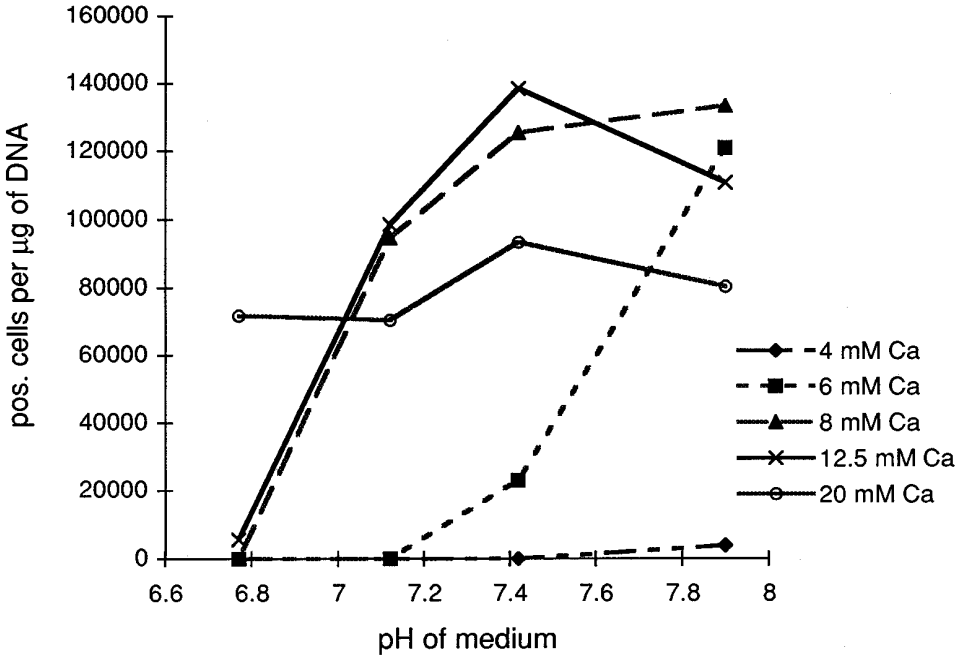

Figure 2. Transfection efficiencies obtained in individual wells expressed as β-gal positive cells per μg of added DNA. HEK-293 cells were exposed to the precipitate under various pH conditions and various calcium concentrations. Cells were fixed for staining with X-gal 1 day after transfection.

Transfections of cells in suspension

Protocols for transfection of suspension cells were modeled after those used for transfections in plates. Cells from an exponentially growing spinner culture were centrifuged and resuspended in fresh medium containing 2% fetal bovine serum at a cell density of about 6×10^5 cells mL^{-1}. In plate experiments, the calcium required to maintain a stable precipitate in cell culture media, is added with the precipitation mixture. For suspension transfections the majority (50–90%) of the required calcium was added (as a 250 mM

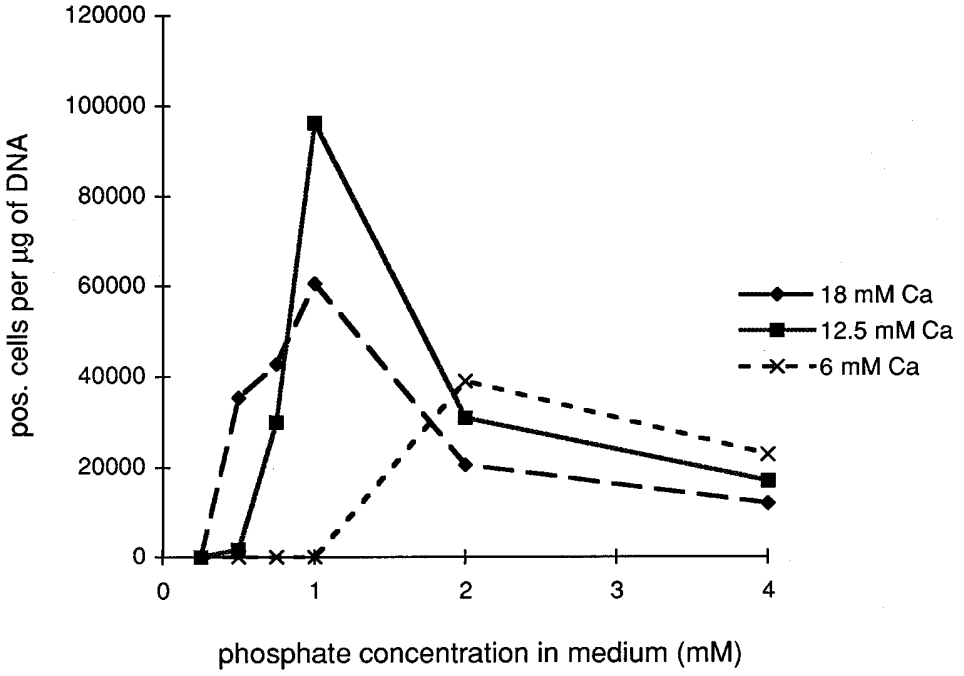

Figure 3. Transfection efficiencies obtained in individual wells of 12-well plates expressed as β-gal positive cells per μg of added. HEK-293 cells were exposed to the precipitate under various phosphate and calcium concentrations.

stock solution) to the cell culture medium immediately before preparing the precipitation mixture. This approach separates the function of calcium during the DNA-calcium phosphate precipitation step from its role to stabilize the precipitate in the cell culture medium.

Extended exposure to a calcium phosphate precipitate can be toxic to cells (data not shown). For this reason, the precipitate has to be removed. In spinners or bioreactors, instead of removing the precipitate by applying a medium exchange, the culture was diluted 1:1 with fresh medium after 3–6 hr. This reduction of the calcium concentration from 12.5 mM to about 6 mM resulted in solubilization of the precipitate. No precipitate could be detected after one hour. In spinner flasks without pH control, the dissolution of the precipitate was further supported by a lower pH which resulted from the metabolic activity of the culture. During the remaining period of the production process, up to 5 days, the cells did not appear to suffer from the exposure to calcium concentrations which were about 6 times higher than in the standard culture media.

Figure 4 shows product titers in spinner flasks one and 2 days after transfection. Cells were directly transfected in 100 or 250 mL flasks with precipitate formed for 1, 5 and 20 min. While there was no notable difference in levels of protein expression between the 100 mL and the 250 mL culture, the precipitate complexes which were allowed to develop during a 1 min reaction time resulted in a 2- to 8-fold higher titer of secreted proteins than those which were derived from a 5 or 20 min reaction time. Essentially the same results were seen in plate experiments, correlating higher titers with shorter incubation times for precipitate complex formation, as previously published (Jordan et al., 1996). This indicates that transfection in suspension is reproducible and not limited to a particular volume if the conditions to form the precipitate have been defined precisely and cells from one batch are used.

The impact of precipitate formation on transfection efficiency

The formation of an efficient precipitate complex can not only be optimized by choosing the appropriate reaction time but also by adjusting the phosphate concentration in the precipitation mixture. In the experiments described below, transfections done with attached cells in 12-well plates are compared with transfections performed with cells in suspension. Using one calcium/DNA solution and a set of HEPES/phosphate solu-

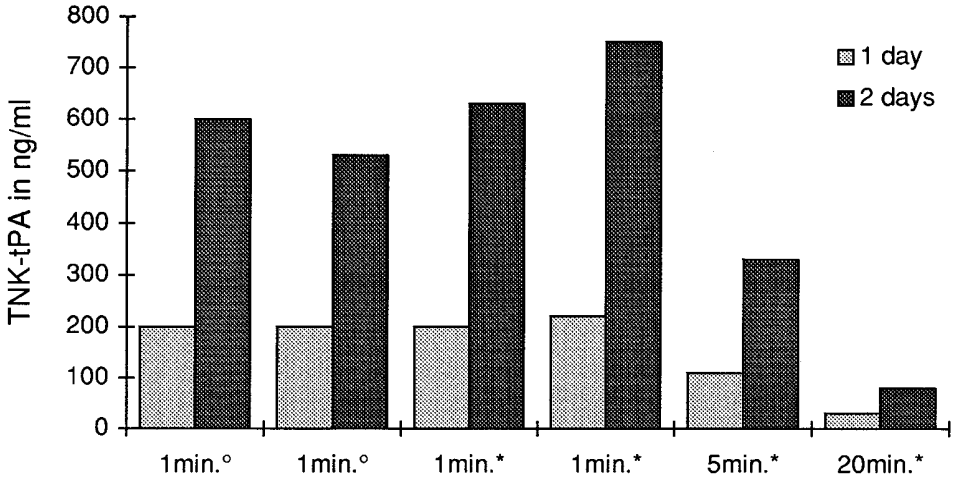

Figure 4. Transient transfection of HEK-293 cells in suspension, cultivated in duplicate in 100 mL (°) and 250 mL (∗) spinner flasks: expression of TNK-tPA after one or two days. The three pairs of columns on the right side give the results from one batch of precipitation mixture added to the culture after 1, 5 and 20 min.

tions to create precipitates of a different nature, titers observed in plates can be compared with those obtained in 250 mL spinner flasks. To study DNA association with the forming precipitate, a parallel sample of each of the precipitation mixtures was spun down, and unbound DNA was measured in the supernatant.

In both transfection systems levels of expression correlated with the respective phosphate concentrations (Figure 5). Phosphate concentrations below 0.5 mM, which did not support the precipitation of DNA, did not result in detectable protein expression. On the other hand, phosphate concentrations higher than 2 mM, which caused a heavy precipitate with relatively large particles, also lead to poor expression. Phosphate concentrations in the range of 0.65–1.5 mM gave product titers of about 0.4 mg L^{-1}. Product titers were quite similar in both systems and it seems that experiments in plates are in general representative for trends of expression in suspension. It has to be mentioned, that for transfections with suspension cells five times less DNA was used (0.5 μg DNA mL^{-1} medium versus 2.5 μg DNA mL^{-1} medium), but more experiments have to be done to address the effects of DNA concentration in the medium.

Effect of pH during incubation of cells and DNA-vehicle

Once an appropriate precipitate has been formed under defined conditions, such a precipitate must be maintained in the cell suspension for a few hours in order to provide efficient DNA-uptake. The pH of the medium would appear to be a good parameter for controlling the stability of the precipitate. The effect on the transfection efficiency already has been shown in plates with adherent cells (Figure 2). The relationship between pH and transfection efficiency has also been investigated by transfecting cells in suspension under controlled pH-conditions in bioreactors. Cells growing as suspensions in a 100 L bioreactor were diluted during the exponential growth phase with fresh medium to a density of 0.3×10^6 cells mL^{-1} and were pumped into 2 L bioreactors. Transfections were started within 2 hr. The pH was shifted from 7.2 to the desired value (4 values between 7.2 and 7.8) 30 min before increasing the calcium concentration. Subsequently, the precipitation mixture was added. Four hours later the pH was readjusted to 7.2 and fresh medium added to reduce the calcium level and to dissolve the remaining precipitate.

At a pH of 7.2, the cell culture medium in the bioreactor during the transfection appeared 'normal'. After 3 days expression, 150 ng recombinant protein per milliliter medium was detected, a value which did not increase between day 3 and 5 (Figure 6A). At a pH of 7.4, a slight cloudiness of the medium was noted 5 min after the injection of the precipitate and, most important, the expression levels were 3 times higher than at pH 7.2. At pH 7.8, the medium became completely cloudy with a high quantity of particles being visible under the light microscope. Despite the high calcium-phosphate concentration and prolonged exposure time of 4 hr to a pH of 7.8, no adverse effects on

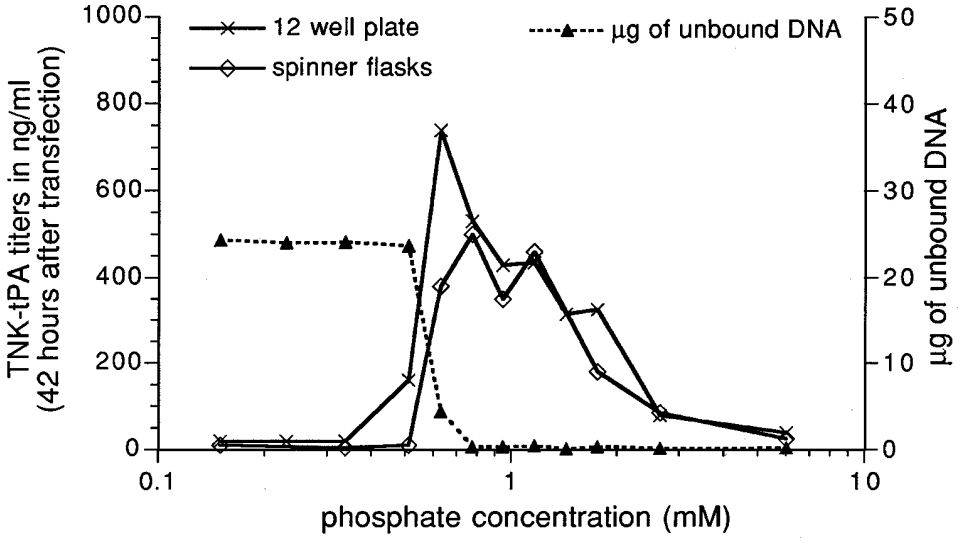

Figure 5. Transient transfection in plates with attached cells versus transfections in spinner flasks with suspension adapted cells: effect of various phosphate concentrations during the precipitation step on binding of DNA and on transient expression of TNK-tPA in 293 cells.

viability or growth of these cells were observed (data not shown). The expression levels, however, were low, reaching only about 100 ng mL^{-1} of TNK-tPA. These results suggest that at pH 7.8 or 7.6 the medium was too saturated with calcium and/or phosphate. On the other hand, at pH 7.2, the concentration of calcium may have been too low to guarantee uptake of DNA.

Nevertheless in a second experiment a pH of 7.2 was used, with the advantage that no pH shift is needed, and 2 further calcium concentrations tested. Reducing the calcium concentration from 12.5 mM to 10 mM during the transfection increased the solubility of the added precipitate, and, as expected, slightly reduced expression levels of TNK-tPA (Figure 6B). An increase of the calcium concentration to 17 mM, on the other hand, generated a more saturated medium, and expression was improved by a factor of 3 over that obtained with 12.5 or 10 mM calcium. This titer is comparable to the case where cells were transfected at pH 7.4 and 12.5 mM calcium. Again, the interplay of calcium concentration and pH becomes obvious, indicating an optimal concentration of calcium for each specific pH. These data show that the calcium phosphate technique is suitable for transfection of cells in suspension. Precipitate particles interact with cells in suspension in a stirred tank and this is sufficient to introduce DNA into cells.

More experimental work is needed to optimize parameters such as DNA concentration and cell density, as well as addressing the possibility of toxic effects. In addition, other cell lines, suitable for transfection with calcium phosphate, should also be tested in suspension. The protocol developed for the 1–2 L scale is, without major changes, adaptable to the 10 L scale.

Conclusion

It was demonstrated that the calcium phosphate transfection method can be adapted and optimized for transfection of cell suspensions. The work presented in this paper used a suspension adapted HEK-293 cell line for which we find reliability and high efficiency of calcium phosphate mediated DNA transfer to cells. The aim of this work was not to develop a standard protocol, but to show that the concept of transfection is applicable to suspension cell culture. It is clear that the maximum transient expression levels in suspension cultures have not yet been attained, however, the results motivated us to investigate transfections at larger scales and with other cell lines.

Using the calcium phosphate technique to transfect cells two general principles were observed: a) the formation of a precipitate of appropriate quality is essential and the desired precipitate consists of a large number of small size particles, which can be observed in a macroscopic scale as a visible, yet fine cloudiness. Such a precipitate contains a large amount of DNA. We estimate a surprisingly high packing capacity of DNA (DNA representing up to 30% of the total weight of the

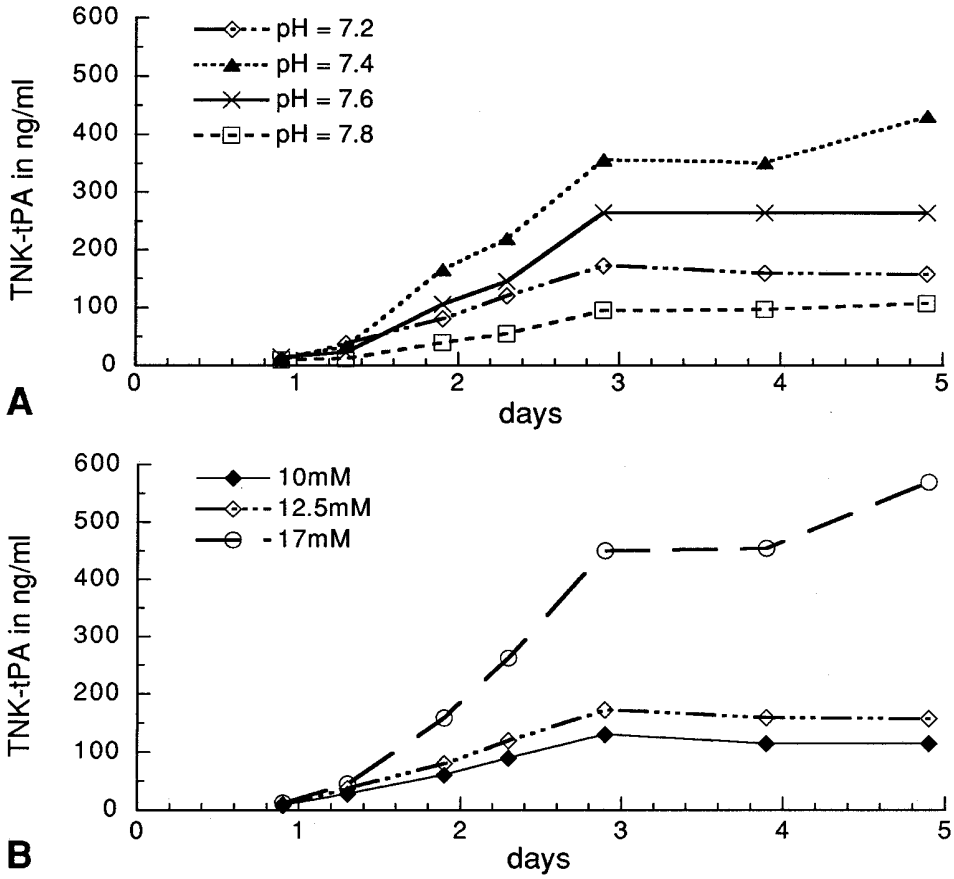

Figure 6. Transient transfection of HEK-293 cells in suspension cultivated in 2 L bioreactors. Experiments were performed in duplicate. Observed titers for TNK-tPA over a period of 5 days were plotted as the average of two parallel transfections. (A) Set-points of pH during the exposure to calcium-phosphate DNA co-precipitates were adjusted for a 4 hr period to pH 7.2, 7.4, 7.6 and 7.8. (B) Three different calcium concentrations (10, 12.5, 17 mm) were studied at a pH of 7.2.

precipitate). b) once a precipitate has been formed and added to the cells, it is not static. The precipitate will undergo pH and calcium or phosphate concentration dependent changes and it will dissolve in non-saturated solutions. We demonstrated that precipitate stability in the medium can be easily manipulated. This is important in order to obtain maximum expression levels in plate cultures. In addition, solubility problems can be avoided when adapting the method to a larger scale. It is possible that the addition of small particles to relatively large medium volumes results in non-saturated conditions initially, which could lead to rapid dissolution of particles before the critical calcium concentration has been achieved. On the other hand, if the degree of over-saturation is too high, particles grow to larger complexes, fueled by an on-going precipitation process in the medium. A pH shift, and/or addition of calcium to the cells, before the precipitate is added is recommended while the phosphate concentration should be maintained constant. Clearly information about calcium phosphate solubility in the medium is essential for reproducible transfection experiments. The issue of solubility within a given environment offers the potential for control of precipitate quality; with a correct adjustment of calcium concentration resulting in transfection at any physiological pH.

With presently obtained transfection efficiencies, yields of up to 20 mg of a recombinant tPA molecule would be expected to be produced in a 10L reactor within just 3 days. On the DNA input level of this scheme, the recovery and purification of 5–10 mg of an expression vector for a novel recombinant protein is fast and can be done with standard protocols. Thus, fast synthesis of significant quantities of novel proteins can

be achieved with this approach, obviating the need for the generation of stable cell lines for production purposes. In this context it should be mentioned that the transient expression of about 60 variant tPA molecules and the subsequent expression of multiple combinations of these mutants in HEK-293 cells provided data for the design of TNK-tPA, a more active plasminogen activator, which is presently in clinical trials. The data, initially obtained with proteins from HEK-293 cells, were in fact predictive for the characteristics of the same tPA-variants, eventually expressed from stable CHO cells (Bennett et al., 1991; Paoni et al., 1993).

It is expected that transient expression in HEK-293 cells can be scaled-up to the 100 L or more and that the ratio of DNA needed to protein produced can be lowered in future, making this technology attractive for many applications in biotechnology.

Acknowledgments

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