Gene transfer effects on various cationic amphiphiles in CHO cells

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Abstract Gene transfer is an important tool to explore genomic, cell biologic, or gene therapeutic research. In this paper we report that several cationic amphiphiles have the potential to efficiently deliver DNA into CHO cells, which is one of the cell lines considered to be important for production of proteins including therapeutic proteins. We have found that *O,O'*-ditetradecanoyl-*N*-(trimethylammonio acetyl) diethanolamine chloride (14Dea2), among 29 types of cationic amphiphiles tested, shows a transfection efficiency of more than 40% in CHO cells. In addition, the results from a series of hydrocarbon chains of varying lengths bound to a connector have shown that an optimal chain length is important for the efficient delivery of DNA into cells. Moreover, flow cytometer analysis has shown that 14Dea2 transfection leads to high levels of expression of the reporter gene (green fluorescent protein) in individual cells. These findings have suggested that 14Dea2 is able to effectively deliver a number of plasmids into a cell nucleus. Thus, our system might be a powerful tool for high

efficiency gene transfer and production of high levels of recombinant protein.

Keywords Gene transfer · Cationic amphiphile · Hydrocarbon chain · CHO cells · GFP expression

Abbreviations

14Dea2 *O,O'*-ditetradecanoyl-*N*-(trimethylammonio acetyl) diethanolamine chloride
GluP Glutamate and phenylene group
GluN Glutamate and naphthalene group

Introduction

Gene transfer techniques in mammalian cells serve as an important tool in genomic, cell biologic, or therapeutic research. They have been developed to utilize liposomes (Schaeffer-Ridder et al. 1982; Felgner et al. 1987), calcium phosphate (Graham and Van der Eb 1973; Chen and Okayama 1987), DEAE dextran (McCutchan and Pagano 1968; Lopata et al. 1984), ballistic delivery (Johnston and Tang 1993), viral systems (Cone and Mulligan 1984), microinjection (Capecchi 1980), and electroporation (Neumann et al. 1982; Chu et al. 1987). Liposome-mediated

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gene transfer is the most widely used method. Actually, several liposome reagents such as Lipofectin, Lipofectamine 2000 (Invitrogen), FuGENE 6 (Roche Applied Science), CLONfectin (Clontech, Takara Bio), or Geneporter (Gene Therapy systems) are commercially available and are used for gene transfer approaches in a number of cell types and tissues. Formation of liposome-DNA complexes protects the DNA and acts as a vehicle that delivers target genes into cells with lower cell toxicity as compared to calcium phosphate, viral vectors, or other systems.

Chinese hamster ovary (CHO) cell is one of the cell lines that is considered highly efficient for the production of proteins including therapeutic proteins. For example, human erythropoietin (Davis et al. 1987; Mori et al. 1994; Lee et al. 1999), human transforming growth factor beta (Derynck et al. 1985; Bourdrel et al. 1993), or human bone morphogenetic protein-2 (Wozney et al. 1988; Israel et al. 1992) have been commercially produced using CHO cells. For these reasons, many studies involving CHO cells have provided a novel approach for the increased synthesis of recombinant proteins (Dorner et al. 1989; Gu et al. 1994), effective expression of foreign genes (Cockett et al. 1991; Lucas et al. 1996; Lacy-Hulbert et al. 2001), stable protein production (Waldman and Waldman 1998; Barnes et al. 2003), or stably transfected cell lines (Perret et al. 1990; Gurtu et al. 1996; Zahn-Zabal et al. 2001). In previous studies, we reported that novel quaternary ammonium amphiphiles have a potential to deliver plasmids including reporter genes into mammalian cells (Ito et al. 1990; Akao et al. 1994). In this study, to further develop our gene transfer system for mammalian cells, we tried to optimize the conditions for the cationic amphiphile-mediated transfection into CHO cells. Here, we found that 14Dea2, among 29 types of cationic amphiphiles tested, has a potential to yield the highest efficiency of gene transfer and high levels of green fluorescent protein (GFP) expression in individual cells. These findings suggest that our system might be useful for achieving a high efficiency of gene transfer and the production of high levels of recombinant protein.

Materials and methods

Cationic amphiphiles and expression vector

The cationic dialkyl ammonium amphiphiles were synthesized as previously described (Kunitake and Okahata 1977; Kunitake et al. 1980; Kunitake 1986). Briefly, 14Dea2 was derived from diethanolamine which was used as a connector to bind two hydrocarbon chains (Fig. 1a). Moreover, 12Glu4 was from glutamine which was used as a connector to bind two hydrocarbon chains. The other amphiphiles—14Asp2, 12GluP2, and 12GluN3—contain asparagine, glutamate and phenylene group, and glutamate naphthalene group, respectively, as a connector of two hydrocarbon chains. For 2C16NC2, the hydrocarbon chains were directly bound to the ammonium nitrogen.

The pCMV-GFP vector (Clontech, Takara Bio) used in all the experiments contains a green fluorescent protein (GFP) as a reporter gene under the control of the cytomegalovirus (CMV) immediate early promoter.

Cell culture and transfection

CHO-K1 cells (Riken BioResource Center) were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with 10% FBS (ICN Biomedicals) at 37°C. For DNA transfection, the cells were plated into 24-well plates at a density of 1.0×10^5 cells per well. Various amphiphile solutions were prepared by ultrasonication for 1.0 min in H₂O at a final concentration of 1.3 mM. The plasmid DNA (1.0 µg) and amphiphile solutions (5.0 µl) were mixed in 150 µl of the medium and then incubated for 5.0 min at room temperature. The cells were washed once with the medium. Subsequently, 150 µl of the medium and 150 µl of amphiphile/DNA mixtures were added, and the cells were incubated for 3.0 h at 37°C. Following transfection, the medium was shifted to fresh DMEM with 10% FBS and GFP-positive cells were detected after 24 h by fluorescence microscopy. For quantitative estimations, fluorescent cells were counted in more than three different views (at least 300 cells per view) by a fluorescence microscope and analyzed by fluorescence-activated cell sorting (FACS)

Fig. 1 Transfection effects on various cationic amphiphiles for CHO cells. **(a)** Structures of the synthetic cationic amphiphiles; 14Dea2, 14Dea6, 14Glu4, 14Glu6, 14Asp2, and 2C16NC2. **(b)** Transfection efficiencies of 29 types of cationic amphiphiles. CHO cells were plated in 24-well plates and incubated for 3.0 h with the amphiphile/DNA mixtures (a ratio of 1:2; 5.0 μ l 1.3 mM amphiphile:1.0 μ g DNA). GFP-positive cells were counted in more than three different views (at least 300 cells per view) at 24 h post transfection by a fluorescence microscope. All data points are averaged from three replications on different days and expressed as the mean $\pm$ standard deviation. The closed column represents transfection efficiency of approximately 15% or more

using an EPICS flow cytometer (Beckman Coulter). Cell viability was measured by using a 0.1% trypan blue dye (Invitrogen) exclusion assay after 24 h transfection.

For calcium phosphate transfection, a coprecipitate of the plasmid (1.0 μ g per well) and calcium phosphate was allowed to form by slowly mixing into HEPES-buffered saline. After incubation for 30 min at room temperature, the precipitate was slowly added to the cells and incubated for 3.0 h before being replaced with 10% FBS-supplemented DMEM.

Trypan blue exclusion assay

Twenty-four hours after transfection, CHO cells treated as indicated were harvested by 0.2%

trypsin–EDTA solution and stained for 5.0 min with 0.1% trypan blue dye solution (Invitrogen). Cell viability was expressed as the percentage of trypan blue-unstained cells. At least 300 cells per treatment were counted under microscope.

Flow cytometry analysis

To estimate the fluorescence intensity of GFP expression in individual cells, CHO cells were harvested using 0.2% trypsin–EDTA solution at 24 h post transfection and 5.0×10^4 events were analyzed on an EPICS flow cytometer with a 488-nm argon laser at 550 V. The high fluorescence intensity of GFP-positive cells was estimated as a range of more than 10^2 FL1 LOG on a flow cytometry histogram.

Results

Transfection efficiencies of various cationic amphiphiles for CHO cells

In the first series of experiments, we tested the various types of cationic amphiphiles to develop a high efficiency of transfection for CHO cells. Cells were incubated for 3.0 h with amphiphile/DNA mixtures, and GFP expression was measured at 24 h post transfection. The transfection mixtures contained a final concentration of 0.02 mM (6.5 nmol) amphiphile and 1.0 μ g DNA, which were mixed so as to obtain a 1:2 ratio of cationic amphiphile charges to anionic DNA charges. We found that six types of amphiphiles (Fig. 1a) showed a transfection efficiency of 15% or more in cells; 14Dea2, $24.3 \pm 2.24\%$; 14Dea6, $14.6 \pm 2.29\%$; 14Glu4, $18.7 \pm 3.21\%$; 14Glu6, $22.2 \pm 3.42\%$; 14Asp2, $18.0 \pm 3.93\%$; and 2C16NC2, $15.7 \pm 3.21\%$ (Fig. 1b). Further, the results from a series of hydrocarbon chains of varying lengths bound to a connector showed that an optimal chain length significantly increases the transfection efficiency; for example, comparisons between the transfection efficiencies of 14Dea2 ($24.3 \pm 2.24\%$) and 12Dea2 (0%), 14Glu4 ($18.7 \pm 2.31\%$) and 14Glu2 ($0.45 \pm 0.19\%$), or 2C16NC2 ($15.7 \pm 3.21\%$) and 2C14NC2 ($0.55 \pm 0.25\%$). Thus, the length of the hydrophobic chain played an important role in gene transfer into cells.

Effects of serum on amphiphile transfection

We examined the effects of serum on the transfection efficiency with 14Dea2, 14Dea6, 14Glu4, 14Glu6, 14Asp2, and 2C16NC2. When CHO cells were incubated for 3.0 h with transfection mixtures containing an increased concentration of serum (1.0–10%), the transfection efficiency was significantly reduced in the presence of 2.5% or more serum, except in the case of 14Dea2 (Fig. 2). In the case of 14Dea2, which gave the highest efficiency among the amphiphiles tested, the addition of less than 2.5% serum scarcely reduced the efficiency. These results showed that 14Dea2 has a significantly higher transfection efficiency on CHO cells than the other amphiphiles.

Fig. 2 Effects of serum on amphiphile-mediated transfection. Cells were plated in 24-well plates and incubated for 3.0 h with the amphiphile/DNA mixtures (a ratio of 1:2) containing the indicated concentrations of serum (0–10%). GFP-positive cells were counted in more than three different views (at least 300 cells per view) at 24 h post transfection by a fluorescence microscope. All data points are averaged from three replications on different days and expressed as the mean $\pm$ standard deviation

Optimization of amphiphile/DNA ratio

The optimal amphiphile/DNA ratio on cationic/anionic charges was examined by varying the DNA concentration and the amphiphile volume. For dose response of 0.2–4.0 μ g DNA per well against 5.0 μ l amphiphile per well, 14Dea2 and 2C16NC2 in the amphiphile/DNA ratio of 3:4 (1.5 μ g DNA per well) yielded the significantly high transfection efficiency, whereas 14Dea6, 14Glu4, 14Glu6, and 14Asp2 showed an optimal ratio of 1:2 (1.0 μ g DNA per well) (Fig. 3a–f). In particular, when cells were incubated for 3.0 h with a 3:4 ratio of 14Dea2/DNA, the efficiency reached the maximal level at $41.3 \pm 2.31\%$ (Fig. 3a). In contrast, when CHO cells were incubated for 3.0 h with 2.5 μ g DNA or more per well, which neutralized the net positive charge of the amphiphile/DNA complexes, the efficiency was significantly reduced. For dose response of 1.0–10 μ l amphiphile per well against 1.0 μ g DNA per well, 14Dea2 at a dose range of 4.0–6.0 μ l showed the higher transfection efficiency from $26.3 \pm 12.9\%$ at 4.0 μ l to $22.4 \pm 5.57\%$ at 6.0 μ l (Fig. 3h), whereas the other amphiphiles showed an following optimal range; 14Dea6, 5.0–7.0 μ l per well; 14Glu4 and 14Glu6, 6.0–8.0 μ l per well; 14Asp2, 7.0–9.0 μ l per well; 2C16NC2, 9.0–10 μ l

Fig. 3 Effects of amphiphile/DNA ratio on amphiphile-mediated transfection. **(a–f)** Transfection efficiencies under various concentrations of DNA (0–4.0 μg per well). Cells were plated in 24-well plates and incubated for 3.0 h with 5.0 μl of 1.3 mM amphiphile and the indicated DNA concentrations. GFP-positive cells were counted in more than three different views (at least 300 cells per view) at 24 h post transfection by a fluorescence microscope. **(g)** Cell viability at dose response of DNA. Cell viability was measured by using trypan blue exclusion assay after 24 h transfection (as described in “Materials and methods”). **(h–m)** Transfection efficiencies under various volumes of

1.3 mM amphiphile solutions (0–10 μl per well). Cells were plated in 24-well plates and incubated for 3.0 h with 1.0 μg DNA and the indicated amphiphile volumes. GFP-positive cells were counted in more than three different views (at least 300 cells per view) at 24 h post transfection by a fluorescence microscope. **(n)** Cell viability at dose response of amphiphile. Cell viability was measured by using trypan blue exclusion assay after 24 h transfection (as described in “Materials and methods”). All data points are averaged from three replications on different days and expressed as the mean ± standard deviation

Fig. 3 continued

per well (Fig. 3i–m). In particular, when cells were incubated for 3.0 h with 7.0 μl 14Glu6 per well, the efficiency reached the higher level at $29.0 \pm 3.80\%$ (Fig. 3k). These results showed that the amphiphile/DNA ratio is important for gene transfer and an optimal ratio of 5.0 μl 14Dea2:1.5 μg DNA has the highest efficacy to enhance gene expression.

Next, we estimated cell toxicity in transfection testing for the dose responses of DNA and amphiphile by using trypan blue staining. Cell viability in transfection with 14Dea2 or 2C16NC2 was $94.0 \pm 3.50\%$ at 5.0 μl 14Dea2:1.0 μg DNA and $96.8 \pm 3.93\%$ at 5.0 μl 2C16NC2:1.0 μg DNA (Fig. 3g), whereas the other amphiphiles showed

99.5 ± 9.85% cell death at 8.0 µl 14Dea6:1.0 µg DNA, 97.9 ± 1.32% at 10 µl 14Glu4:1.0 µg DNA, 97.5 ± 2.20% at 9.0 µl 14Glu6:1.0 µg DNA, and 94.5 ± 5.20% at 10 µl 14Asp2:1.0 µg DNA (Fig. 3n). Thus, the trypan blue exclusion analysis showed that transfection mediated by six types of amphiphiles had low cell toxicity to CHO cells and more than 90% cells were viable irrespective of the transfection treatment.

Optimization of transfection time for 14Dea2

We found that 14Dea2 has the highest transfection efficiency in CHO cells. In order to determine the optimal time for gene transfer with 14Dea2, cells were incubated for different time intervals (0.2–6.0 h) with the 14Dea2/DNA mixtures (a ratio of 3:4) and the transfection efficiency after 24 h was measured. As shown in Fig. 4, the efficiency was increased from 6.55 ± 1.03% at 0.5 h, saturated at 3.0 h (40.3 ± 1.55%), and reached 47.4 ± 1.89% at 6.0 h. Similar results were obtained from the other amphiphile-mediated transfection, namely, with 14Dea6, 14Glu4, 14Glu6, 14Asp2, and 2C16NC2 (data not shown). Thus, our amphiphiles, as demonstrated by 14Dea2, required at least 3.0 h incubation for gene transfer.

14Dea2 enhances the number of cells with high GFP expression level

Transfection techniques for the high expression of target proteins in mammalian cells are efficient tools to establish a production system for recombinant proteins. We measured the fluorescence intensity of GFP expression in individual cells transfected with six types of amphiphiles by a flow cytometry analysis (488-nm argon laser at 550 V). When cells were incubated for 3.0 h with the indicated amphiphile/DNA mixtures, 14Dea2 dramatically gave rise to a greater number of cells (28.9 ± 3.39%) in the range of high fluorescence intensity (shown as “High” on a flow cytometry histogram) as compared with 14Dea6 (7.78 ± 1.09%), 14Glu4 (8.73 ± 0.91%), 14Glu6 (15.8 ± 1.71%), 14Asp2 (8.27 ± 0.95%), and 2C16NC2 (9.45 ± 1.27%) (Fig. 5a–g). In addition,

Fig. 4 Effects of incubation time on 14Dea2-mediated transfection. **(a)** Transfection efficiencies under different time interval (0–6.0 h). Cells were plated in 24-well plates and incubated for the indicated time with the 14Dea2/DNA mixtures (a ratio of 3:4; 5.0 µl 1.3 mM 14Dea2:1.5 µg DNA). GFP-positive cells were counted in more than three different views (at least 300 cells per view) at 24 h post transfection by a fluorescence microscope. All data points are averaged from three replications on different days and expressed as the mean ± standard deviation. **(b)** Fluorescence image of GFP-positive cells (46.2 ± 5.10%). Cells were incubated for 5.0 h with the 14Dea2/DNA mixture (a ratio of 3:4) and then cultured for 24 h in DMEM with 10% FBS: left, phase contrast image (100 ×); right, fluorescence image (100 ×)

we examined the efficiency of calcium phosphate-mediated transfection in CHO cells. When cells were incubated for 3.0 h with calcium phosphate/DNA mixtures in DMEM containing 10% FBS, the efficiency was 0.35 ± 0.19% in the range of high fluorescence intensity (Fig. 5h) and 2.12 ± 0.19% of the total number of GFP-positive cells (data not shown). In contrast with 14Dea2, transfection with calcium phosphate did not induce high levels of GFP expression in individual cells. Thus, 14Dea2 has a potential to effectively deliver a large number of plasmids into individual cells since the high levels of GFP expression were driven by the number of intracellular GFP genes. These findings have shown that 14Dea2 is a powerful tool to achieve both high efficiency of transfection and high expression of foreign genes.

Fig. 5 Flow cytometry analysis of amphiphile-mediated transfection. Cells were plated in 24-well plates and incubated for 3.0 h with the amphiphile/DNA mixtures or calcium phosphate/DNA mixtures. Cells were harvested by 0.02% trypsin solution at 24 h post transfection and analyzed GFP expression by a flow cytometer (5.0×10^4 events, 488 nm excitation wavelength laser, 550 V). The amphiphile/DNA ratio was 3:4 for 14Dea2 and 2C16NC2 or 1:2 for 14Dea6, 14Glu4, 14Glu6, and 14Asp2. A range of high fluorescence intensity, which is shown as “High” on each histogram, was estimated as an amount of more than 10^2 FL1 LOG values. The total percentages in the range of “High” were the following. (a) Untreated, 0%, (b) 14Dea2, $28.9 \pm 3.39\%$, (c) 14Dea6, $7.78 \pm 1.09\%$, (d) 14Glu4, $8.73 \pm 0.91\%$, (e) 14Glu6, $15.8 \pm 1.71\%$, (f) 14Asp2, $8.27 \pm 0.95\%$, (g) 2C16NC2, $9.45 \pm 1.27\%$, and (h) calcium phosphate, $0.35 \pm 0.19\%$. All data points are averaged from three replications on different days and expressed as the mean $\pm$ standard deviation

Discussion

In this paper, we have demonstrated that our cationic amphiphile-mediated gene transfer system is a powerful method to introduce and express a foreign gene (GFP) in CHO cells. The liposome is able to spontaneously interact with DNA, fuse with cellular membranes, and facilitate the delivery of functional DNA into the cells. Among the cationic amphiphiles examined, a cationic liposome formed by 14Dea2 (*O,O'*-ditetradecanoyl-*N*-(trimethylammonio acetyl) diethanolamine chloride) had the highest transfection efficiency for CHO cells. The chemical structure of 14Dea2 (Fig. 1a) may be optimal to deliver DNA to cells. Interestingly, the lengths of the hydrocarbon chains included in the amphiphiles were significantly important in the delivery of DNA into cells. As shown in Fig. 1b, we found a dramatic difference between the transfection efficiencies of 14Dea2 and 12Dea2. The removal of two hydrocarbon chains from 14Dea2 led to an almost complete loss of transfection ability. It appears that optimal lengths of the hydrocarbon chains are important for the construction of amphiphile/DNA complexes that effectively deliver DNA into mammalian cells.

The use of cationic liposome has several advantages, for example, low cytotoxicity, simple preparation, safety, stability, storage, and wide applicability in various cell types. In fact, our cationic amphiphiles had high stability on storage at room temperature, low cytotoxicity, simple method of preparation, and high efficiency of gene transfer. In this study, several parameters were examined to optimize the transfection efficiency. We found that the optimal 14Dea2/DNA ratio of 3:4, which is cationic amphiphile charges to anionic DNA charges, gave rise to 41.3% or a higher efficiency in CHO cells (Fig. 3a) and low cell toxicity of $2.88 \pm 2.72\%$ (Fig. 3g). These results suggest that 14Dea2-mediated transfection is very useful as a research tool for gene transfer in mammalian cells. Interestingly, neutralization of the net positive charge of the amphiphile/DNA complexes led to a significant reduction in the transfection efficiency. This finding suggests that the net positive charge of the amphiphile/DNA

complexes might be an important property for the initial interaction with the negative surface charge of cellular membranes.

It is important to understand gene transfer in CHO cells since they are a widely used cell line for the production of recombinant proteins such as human erythropoietin (Mori et al. 1994; Lee et al. 1999), human transforming growth factor beta (Bourdrel et al. 1993), human bone morphogenetic protein-2 (Israel et al. 1992), or monoclonal antibodies (Rüker et al. 1991; Page and Sydenham 1991; Ierino et al. 1993). In this paper, we have shown that 14Dea2-mediated transfection is able to induce high levels of GFP expression in individual cells in contrast with the other amphiphiles or calcium phosphate (Fig. 5). The results have shown that 14Dea2 has the potential to deliver a great number of plasmids that introduce foreign genes into a cell nucleus and to enhance the protein production in individual cells. Our gene transfer techniques could be very useful not only for high efficiency of gene transfer but also for high expression to establish a cell clone available for high production of recombinant proteins.

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References

- Akao T, Nakayama T, Takeshita K, Ito A (1994) Design of a new cationic amphiphile with efficient DNA-transfection ability. *Biochem Mol Biol Int* 34:915–920
- Barnes LM, Bentley CM, Dickson AJ (2003) Stability of protein production from recombinant mammalian cells. *Biotechnol Bioeng* 81:631–639
- Bourdrel L, Lin CH, Lauren SL, Elmore RH, Sugarman BJ, Hu S, Westcott KR (1993) Recombinant human transforming growth factor-beta 1: expression by Chinese hamster ovary cells, isolation, and characterization. *Protein Expr Purif* 4:130–140
- Capecchi MR (1980) High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell* 22:479–488
- Chen C, Okayama H (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* 7:2745–2752
- Chu G, Hayakawa H, Berg P (1987) Electroporation for the efficient transfection of mammalian cells with DNA. *Nucleic Acids Res* 15:1311–1326

- Cockett MI, Bebbington CR, Yarranton GT (1991) The use of engineered E1A genes to transactivate the hCMV-MIE promoter in permanent CHO cell lines. *Nucleic Acids Res* 19:319–325
- Cone RD, Mulligan RC (1984) High-efficiency gene transfer into mammalian cells: generation of helper-free recombinant retrovirus with broad mammalian host range. *Proc Natl Acad Sci USA* 81:6349–6353
- Davis JM, Arakawa T, Strickland TW, Yphantis DA (1987) Characterization of recombinant human erythropoietin produced in Chinese hamster ovary cells. *Biochemistry* 26:2633–2638
- Derynck R, Jarrett JA, Chen EY, Eaton DH, Bell JR, Assoian RK, Roberts AB, Sporn MB, Goeddel DV (1985) Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. *Nature* 316:701–705
- Dorner AJ, Wasley LC, Kaufman RJ (1989) Increased synthesis of secreted proteins induces expression of glucose-regulated proteins in butyrate-treated Chinese hamster ovary cells. *J Biol Chem* 264:20602–20607
- Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringolds GM, Danielsen M (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci USA* 84:7413–7417
- Graham FL, Van der Eb AJ (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456–467
- Gu MB, Todd P, Kompala DS (1994) Analysis of foreign protein overproduction in recombinant CHO cells. Effect of growth kinetics and cell cycle traverse. *Ann NY Acad Sci* 721:194–207
- Gurtu V, Yan G, Zhang G (1996) IRES bicistronic expression vectors for efficient creation of stable mammalian cell lines. *Biochem Biophys Res Commun* 229:295–298
- Ierino FL, Powell MS, McKenzie IF, Hogarth PM (1993) Recombinant soluble human Fc gamma RII: production, characterization, and inhibition of the Arthus reaction. *J Exp Med* 178:1617–1628
- Israel DI, Nove J, Kerns KM, Moutsatsos IK, Kaufman RJ (1992) Expression and characterization of bone morphogenetic protein-2 in Chinese hamster ovary cells. *Growth Factors* 7:139–150
- Ito A, Miyazoe R, Mitoma I, Akao T, Osaki T, Kunitake T (1990) Synthetic cationic amphiphiles for liposome-mediated DNA transfection. *Biochem Int* 22:235–241
- Johnston SA, Tang DC (1993) The use of microparticle injection to introduce genes into animal cells in vitro and in vivo. *Genet Eng* 15:225–236
- Kunitake T (1986) Organization and functions of synthetic bilayers. *Ann NY Acad Sci* 471:70–82
- Kunitake T, Okahata Y (1977) A totally synthetic bilayer membrane. *J Am Chem Soc* 99:3860–3871
- Kunitake T, Nakanishi N, Shimonura M, Okahata Y, Kano K, Ogawa T (1980) Unique properties of chromophore-containing bilayer aggregates: enhanced chirality and photochemically induced morphological change. *J Am Chem Soc* 102:6642–6644
- Lacy-Hulbert A, Thomas R, Li XP, Lilley CE, Coffin RS, Roes J (2001) Interruption of coding sequences by heterologous introns can enhance the functional expression of recombinant genes. *Gene Ther* 8:649–653
- Lee GM, Kim EJ, Kim NS, Yoon SK, Ahn YH, Song JY (1999) Development of a serum-free medium for the production of erythropoietin by suspension culture of recombinant Chinese hamster ovary cells using a statistical design. *J Biotechnol* 69:85–93
- Lopata MA, Cleveland DW, Sollner-Webb B (1984) High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. *Nucleic Acids Res* 12:5707–5711
- Lucas BK, Giere LM, DeMarco RA, Shen A, Chisholm V, Crowley CW (1996) High-level production of recombinant proteins in CHO cells using a dicistronic DHFR intron expression vector. *Nucleic Acids Res* 24:1774–1779
- McCutchan JH, Pagano JS (1968) Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextra. *J Natl Cancer Inst* 41:351–357
- Mori T, Yamamoto K, Ohta T, Sakamoto C, Sato M, Koide K, Murakami T, Fujii M, Fukuda S, Kurimoto M (1994) A high-level and regulatable production system for recombinant glycoproteins using a human interferon-alpha promoter-based expression vector. *Gene* 144:289–293
- Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider PH (1982) Gene transfer into mouse lymphoma cells by electroporation in high electric fields. *EMBO J* 1:841–845
- Page MJ, Sydenham MA (1991) High level expression of the humanized monoclonal antibody Campath-1H in Chinese hamster ovary cells. *Biotechnology* 9:64–68
- Perret J, Ludgate M, Libert F, Gerard C, Dumont JE, Vassart G, Parmentier M (1990) Stable expression of the human TSH receptor in CHO cells and characterization of differentially expressing clones. *Biochem Biophys Res Commun* 171:1044–1050
- Rüker F, Ebert V, Kohl J, Steindl F, Riegler H, Kättinger H (1991) Expression of a human monoclonal anti-HIV-1 antibody in CHO cells. *Ann NY Acad Sci* 646:212–219
- Schaeffer-Ridder M, Wang Y, Hofschneider PH (1982) Liposomes as gene carriers: efficient transformation of mouse L cells by thymidine kinase gene. *Science* 215:166–168
- Waldman AS, Waldman BC (1998) Stable transfection of mammalian cells by syringe-mediated mechanical loading of DNA. *Anal Biochem* 258:216–222
- Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA (1988) Novel regulators of bone formation: molecular clones and activities. *Science* 242:1528–1534
- Zahn-Zabal M, Kober M, Girod PA, Imhof M, Chatellard P, de Jesus M, Wurm F, Mermod N (2001) Development of stable cell lines for production or regulated expression using matrix attachment regions. *J Biotechnol* 87:29–42