

## A Novel Transfection Method for Mammalian Cells Using Calcium Alginate Microbeads

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**The direct transfer of genetic materials into mammalian cells is an indispensable technique. We have developed calcium alginate (CA) microbeads which can deliver plasmid DNAs and yeast artificial chromosomes into plant and yeast cells. In this paper, we demonstrate the effective transfection of mammalian cells by CA microbeads immobilizing plasmid DNAs. The transfection was performed using the pEGFP-C1 plasmid containing the cytomegalovirus (CMV) promoter and enhanced green fluorescent protein (EGFP) gene. The transient expression of EGFP was observed 24 h after transfection. The expression efficiency was maximum when the concentration of sodium alginate was 1% and the amount of plasmid DNA was increased to 100 µg. The expression efficiency of our method using CA microbeads is 2–10 times higher than that of the polyethylene glycol (PEG) method. Our results suggest that the CA microbead mediated transfection of mammalian cells effectively delivers genetic materials into mammalian suspension cells.**

[**Key words:** transfection, mammalian cells, calcium alginate, microbeads, plasmid DNA]

The direct transfer of genetic material into mammalian cells is an indispensable technique for the investigation of both gene function and gene therapy. A variety of methods have been reported for the transfection of mammalian cells including microinjection (1), particle bombardment (2), the calcium phosphate method (3), the liposome method (4) and the virus-mediated method (5). However, there are limitations in every technique, with some methods requiring complicated procedures or expensive equipment or having low transfection efficiencies with suspension cells.

Alginate is a harmless polysaccharide and gels in the presence of divalent cations, such as calcium. Alginate is used to immobilize bacterial cells in bioreactors and to encapsulate plant somatic embryos as artificial seeds (6). An emulsion of water in oil (W/O) type is generated by mixing a sodium alginate solution with an organic solvent. Calcium ions were subsequently added to this emulsion and mixed by sonication to solidify the alginate into small gel beads. If hydrophilic materials such as DNA molecules, chromosomes and/or some types of proteins are added to the calcium ion solution, they are occasionally encapsulated into and/or onto the small calcium alginate beads. Transforming growth factor beta 1 was successfully delivered into rat stem cells using alginate beads (7).

We have previously developed calcium alginate microbeads (CA microbeads) and used them in the transfection of yeast and plant cells (8–10). Tobacco BY-2 cells were more effectively transfected using CA microbeads containing plas-

mid DNAs than by the conventional polyethylene glycol (PEG)-mediated method (8, 10). Yeast cells were also successfully transfected using CA microbeads with yeast split chromosome DNA to a maximum size of 450 kb (9). In this paper, we demonstrate that CA microbeads can be applied to the transfection of mammalian cells.

### MATERIALS AND METHODS

**Cell culture** Human lymphocyte cell line K562 cells were grown and maintained at 37°C in humidified 5% CO<sub>2</sub> in RPMI1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen), 100 units/ml of penicillin G potassium salt and 100 µg/ml of streptomycin sulfate (Nakarai Tesque, Kyoto). Human carcinoma cell line HeLa cells were grown and maintained at 37°C in humidified 5% CO<sub>2</sub> in Dulbecco's modified Eagle medium (DMEM; Invitrogen) containing 5% FBS, 100 units/ml of penicillin G potassium salt and 100 µg/ml of streptomycin sulfate.

**Amplification and purification of plasmid** The pEGFP-C1 (Clontech, Palo Alto, CA, USA) plasmid was amplified in the *Escherichia coli* strain DH10B (Invitrogen) and purified using the Quantum Prep plasmid Maxi Prep Kit (Bio-Rad, Hercules, CA, USA). Purity of the plasmid DNA was confirmed by the OD<sub>260</sub>/OD<sub>280</sub> ratio and the intensity of the corresponding DNA fragment upon gel electrophoresis following treatment of the plasmid DNA with restriction enzymes. The concentration of the plasmid DNA was determined using the ratio that 1 (OD<sub>260</sub>) was equivalent to 50 µg of DNA.

**Preparation of CA-microbeads** Isoamyl alcohol (900 µl; Wako Pure Chemicals, Osaka) and 100 µl of sodium alginate (Wako Pure Chemicals) solution were mixed and emulsified by sonication. We defined this amount of sodium alginate solution as

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a standard. A 500- $\mu$ l aliquot of 100 mM calcium chloride solution containing plasmid DNA (pEGFP-C1) was then added to the emulsion to solidify the alginate. The CA microbeads were collected by centrifugation and resuspended in 100 mM calcium chloride solution. This washing step was repeated four times. Finally, the CA microbeads were suspended in 50  $\mu$ l of distilled water. Immobilization of plasmid DNA on the surface of CA microbeads was confirmed by staining microbeads with YOYO-1 (Molecular Probes, Eugene, OR, USA) and observing them under a fluorescence microscope.

**In vitro transfection** Plasmid DNAs were introduced into cells by the PEG-mediated method. PEG treatment was carried out by the procedure described in a previous report (11). Cells at log phase were harvested and a cell suspension of  $2.0 \times 10^6$  cells/ml was prepared. A 500- $\mu$ l aliquot of the cell suspension and 50  $\mu$ l of CA microbeads with plasmid DNAs were mixed. A 0.5-ml aliquot of a 16% PEG4000 (Wako Pure Chemicals) solution (16% PEG4000, 0.8 M sucrose, 170 mM NaCl, 50 mM Tris-HCl [pH 7.3], 20% dimethyl sulfoxide) was then added and the mixture was incubated for 10 min. RPMI1640 medium without FBS (1 ml) was added and the mixture further incubated for 5 min, after which 2 ml of RPMI1640 medium without FBS were added and a further 5 min incubation was carried out ( $\times 2$ ). Cells were harvested by centrifugation at  $190 \times g$  for 5 min and resuspended in 5 ml of RPMI1640 medium (10% FBS added) and cultivated at 37°C, in 5% CO<sub>2</sub> for 24 h. When HeLa cells were transfected, DMEM was used instead of RPMI1640 medium.

**Gene expression analysis** After incubation in 5% CO<sub>2</sub> for 24 h, transfected cells were observed by fluorescence microscopy. Fluorescence microscopy was carried out using an inverted IX 71 microscope (Olympus, Tokyo) with a green fluorescence protein (GFP) fluorescent filter. The efficiency of transient expression was calculated as the number of cells emitting enhanced green fluorescent protein (EGFP) fluorescence divided by the total number of

cells.

## RESULTS AND DISCUSSION

### Transfection of mammalian cells using CA microbeads

CA microbeads were prepared by the procedure described above. Transfection was carried out using CA microbeads with polyethylene glycol. Human lymphocyte K562 cells and human carcinoma HeLa cells were transfected using CA microbeads containing 50  $\mu$ g of the plasmid DNA. To confirm transfection, plasmid pEGFP-C1 containing the EGFP gene was used in this study and the transient expression of EGFP was imaged by fluorescence microscopy after 24 h of incubation at 37°C, under 5% CO<sub>2</sub> (Fig. 1). These results suggest that CA microbeads are applicable for the transfection of mammalian cells. As a control, 50  $\mu$ g of non-immobilized plasmid DNA was used for transfection instead of plasmid DNA immobilized on CA microbeads.

We compared the efficiency of transient expression between the PEG method using non-immobilized plasmid DNA and the optimized CA microbead transfection method. The efficiency of the transient expression of the CA microbead-mediated transfection was 2–10 times higher than that of the PEG-mediated method using non-immobilized plasmid DNA (Figs. 2–5).

**Optimization of conditions for transfection** According to our previous experiments, CA microbeads containing DNA molecules are produced efficiently in a 0.5–2.0% sodium alginate solution (8). Because the concentration of sodium alginate affects the physical characteristics of CA microbeads, we optimized the concentration of sodium algi-

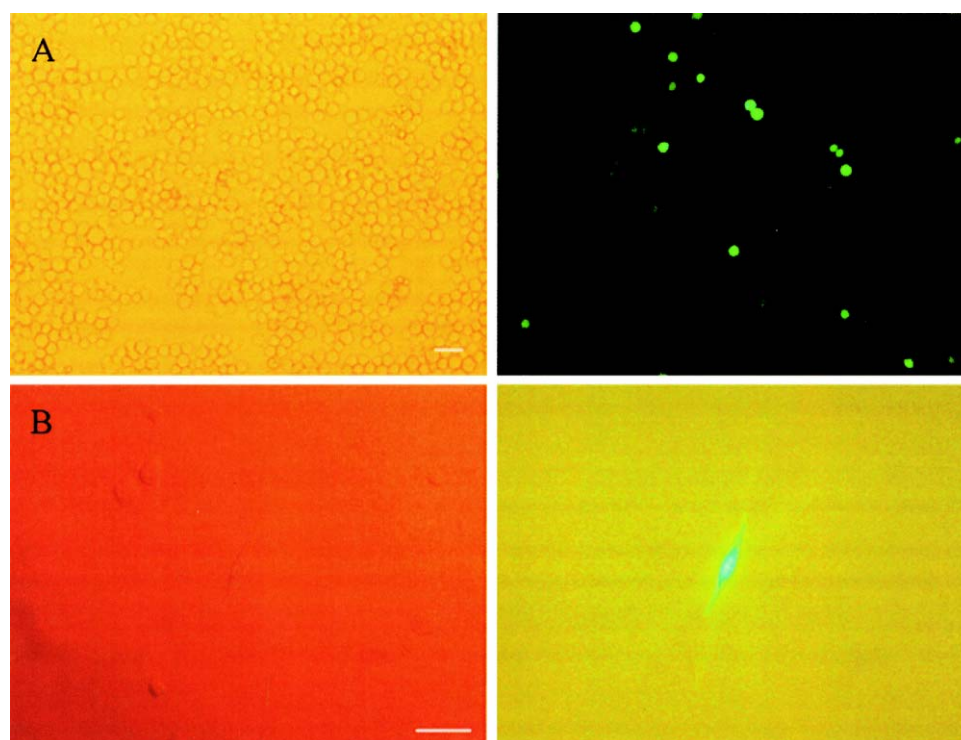


FIG. 1. Expression of EGFP in mammalian cells transfected by CA microbeads. (A) K562 cells. (B) HeLa cells. Left: an image generated by phase contrast microscopy. Right: a fluorescence image generated using a GFP filter. Bars represent 20  $\mu$ m.

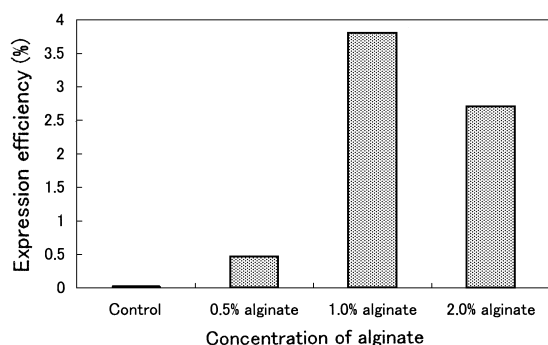


FIG. 2. Optimization of sodium alginate concentration. Preparation of CA microbeads and transfection of K562 cells were performed as described in Materials and Methods except for concentration of sodium alginate.

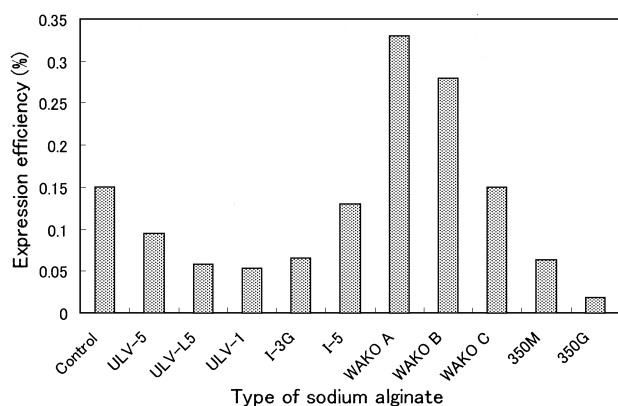


FIG. 3. Effect of sodium alginate viscosity of CA microbeads on expression efficiency. Ten different types of sodium alginate were used for the preparation of CA microbeads. The concentration of sodium alginate was adjusted to 1.0%.

nate for mammalian cells (Fig. 2). The CA microbeads produced from a 1.0% sodium alginate solution showed the most efficient transfection of mammalian cells. Therefore, this concentration of sodium alginate was used in all the experiments for further optimization.

Alginate is a polysaccharide purified from brown seaweed, and the physical and chemical characteristics of alginate beads depend on the place of production and the type of brown seaweed used. Therefore, we purchased and determined the effects of different types of alginate (Wako Pure Chemicals; Kimitsu Chemical Industries, Tokyo; Kibun Food Chemica, Tokyo) on the transient expression efficiency. We examined 10 types of sodium alginate solution, and the solution with a viscosity of 100–150 cP (1% solution, 20°C) resulted in the highest transient expression efficiency of mammalian cells (Fig. 3 and Table 1).

We next optimized the amount of CA microbeads. Using the same amount of DNA molecules for the production of CA microbeads, we determined that the transient expression efficiency was dependent on the amount of CA microbeads used. However, the transient expression efficiency reached a plateau when the amount of CA microbeads reached  $\times 4$  that is the amount of CA microbeads made from 400  $\mu$ l of 1.0% sodium alginate solution (Fig. 4). These results sug-

TABLE 1. Physical characteristics of alginate

Kind of alginate	Condition (20°C)	Viscosity (cP)
ULV-5 (Kimitsu)	10%	500–600
ULV-L5 (Kimitsu)	10%	30–60
ULV-1 (Kimitsu)	10%	100–200
I-3J (Kimitsu)	1%	320–380
I-5 (Kimitsu)	1%	500–600
WAKO A (Wako)	1%	100–150
WAKO B (Wako)	1%	300–400
WAKO C (Wako)	1%	500–600
350M (Kibun)	1%	341
350G (Kibun)	1%	308

cP represents an unit of viscosity of the solution.

1 cP =  $10^{-3}$  kg/m $\cdot$ s

gest that there is a limited amount of plasmid DNA molecules immobilized on the surface of CA microbeads and hence a limited number of CA microbeads adhering to the mammalian cell surface.

Finally, we optimized the amount of plasmid DNA (Fig. 5). The use of 10 to 100  $\mu$ g of plasmid DNA resulted in transient expression efficiency proportional to the amount of plasmid DNA.

Our results clearly indicate that the transient expression efficiency of CA microbead-mediated transfection is significantly higher than that of PEG-mediated transfection and DEAE-dextran method (11) using non-immobilized plasmid DNA (Figs. 2–5). Transfection by electroporation is widely used for mammalian cells in suspension, such as K562 cells. In a previous report, the rate of introduction of plasmid DNA into K562 cells by electroporation was about 8% (12). Although this rate is more or less similar to ours, the CA microbead-mediated method requires no specialized equipment. Furthermore, our method can be used to introduce larger genetic molecules, such as yeast artificial chromosome (YAC) (9). This efficiency improvement is primarily attributed to a higher concentration of DNA molecules at the surface of target cells (13). Our previous report showed that yeast spheroplasts could attach to CA microbeads (9). The physical attachment between cells and CA microbeads might be due to the negative charge of the surface of CA microbeads. Although lipofection method is a powerful method for the introduction of plasmid DNAs into adherent mammalian cells, the transfection efficiency of suspension cells is generally low. Suspension cells could be efficiently transfected by our method, and another advantage of our method is that transfection could be carried out in the presence of serum.

When we used the CA microbeads produced from 1.0% sodium alginate solution, the highest transient expression efficiency was obtained. In previous studies, it was found that the concentration of the sodium alginate solution affects the physical characteristics of CA microbeads (8). CA microbeads produced from a low concentration of sodium alginate tend to be fragile, and therefore might not effectively preserve DNA molecules. On the other hand, CA microbeads produced from a high concentration of sodium alginate might not immobilize the plasmid DNA on their surface because of their high viscosity.

Our results obtained to date demonstrate that mammalian cells can be transfected using calcium alginate microbeads

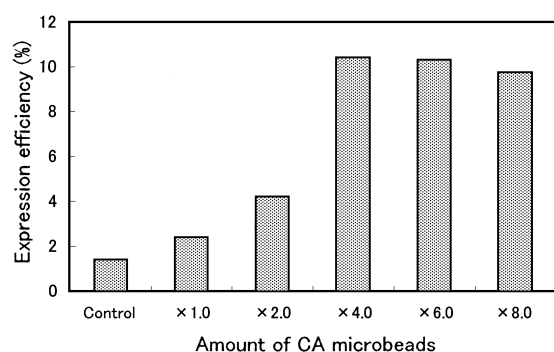


FIG. 4. Effect of the amount of CA microbeads on expression efficiency. The amount of CA microbeads was changed according to the amount of sodium alginate.  $\times 1$  indicates the standard amount of sodium alginate for producing CA microbeads as described in Materials and Methods.

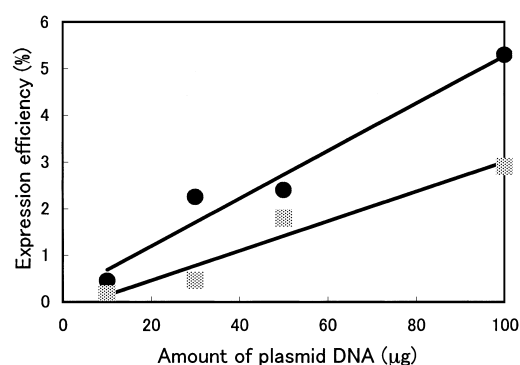


FIG. 5. Effect of the amount of plasmid DNAs on expression efficiency. K562 cells were transfected using CA microbeads containing various amounts of plasmid DNA (circles) and various amounts of non-immobilized plasmid DNA (squares).

containing plasmid DNA molecules and that gel-purified chromosomal DNA (260 and 280 kb) can be stably immobilized on CA microbeads (9). In recent years, a range of viral vectors that have the potential to package large-sized inserts have been developed (14–17). However, the use of viral vectors for transferring the genetic materials into cells has associated safety issues including the spread of viruses, oncogene activity and immuno-suppression. Our calcium alginate microbead-mediated method might be suitable for delivering a large amount of genetic information into mammalian cells given that yeast cells can be successfully transformed by chromosome-containing CA microbeads (9). It is anticipated that in the near future we will successfully transfect mammalian cells with larger DNA molecules, such as the human artificial chromosomes (HACs) or mammalian artificial chromosomes (MACs) using the CA microbead-mediated method. HACs containing the guanosine triphosphate cyclohydrolase 1 (CGH1) gene were generated and their stable maintenance in human and mouse cell lines was reported (18). Therefore these HACs could be used as good vectors for introducing large DNA fragments containing genes and their complete endogenous promoter regions into mammalian cells.

In conclusion, the CA microbead method will likely be-

come a good alternative for the transfection of mammalian cells.

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## REFERENCES

1. Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., and Felgner, P. L.: Direct transfer into mouse muscle *in vivo*. *Science*, **247**, 1465–1468 (1990).
2. Yang, N. S., Burkholder, J., Robert, B., Martinell, B., and McCabe, D.: *In vivo* and *in vitro* gene transfer to mammalian somatic cells by particle bombardment. *Proc. Natl. Acad. Sci. USA*, **87**, 9568–9572 (1990).
3. Benvenisty, N. and Reshel, L.: Direct introduction of genes into rats and expression of the genes. *Proc. Natl. Acad. Sci. USA*, **83**, 9551–9555 (1986).
4. Felgner, P. L., Gadak, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., and Danielsen, M.: Lipofection: a highly efficient, lipid-mediated DNA-transfer procedure. *Proc. Natl. Acad. Sci. USA*, **84**, 7413–7417 (1987).
5. Akli, S., Cailaud, C., Vigne, E., Stratford-Perricaudet, L. D., Poenaru, L., Perricaudet, M., Kahn, A., and Peschanski, M. R.: Transfer of foreign gene into the brain using adenovirus vectors. *Nat. Genet.*, **3**, 224–228 (1993).
6. Kersulec, A., Bazinet, C., Corbinau, F., Come, D., Barbotin, J. N., Hervagault, J. F., and Thomas, D.: Physiological behavior of encapsulated somatic embryos. *Biomater. Artif. Cells Immobilization Biotechnol.*, **21**, 375–381 (1993).
7. Oulakkainen, P. A., Ranchalis, J. E., Gombotz, W. R., Hoffman, A. S., Mumper, R. J., and Twardzik, D. R.: Novel delivery system for inducing quiescence in internal stem cells in rats by transforming growth factor beta 1. *Gastroenterology*, **107**, 1319–1326 (1994).
8. Sone, T., Nagamori, E., Ikeuchi, T., Mizukami, A., Takakura, Y., Kajiyama, S., Fukusaki, E., Harashima, S., Konayashi, A., and Fukui, K.: A novel gene delivery system in plants with calcium alginate micro-beads. *J. Biosci. Bioeng.*, **94**, 87–91 (2002).
9. Mizukami, A., Nagamori, E., Takakura, Y., Matsunaga, S., Kaneko, Y., Kajiyama, S., Harashima, S., Kobayashi, A., and Fukui, K.: Transformation of yeast using calcium alginate microbeads with surface-immobilized chromosomal DNA. *BioTechniques*, **35**, 734–740 (2003).
10. Liu, H., Kawabe, A., Matsunaga, S., Murakawa, T., Mizukami, A., Yanagisawa, M., Nagamori, E., Harashima, S., Kobayashi, A., and Fukui, K.: Obtaining of transgenic plants using the bio-active beads method. *J. Plant Res.* (2004). (in press)
11. Takai, T. and Ohmori, H.: Highly efficient DNA transfection of hematopoietic cell lines by an improved DEAE-dextran method. *Methods Mol. Cell. Biol.*, **2**, 82–90 (1990).
12. Takahashi, M., Furukawa, T., Saitoh, H., Aoki, A., Koike, T., Moriyama, Y., and Shibata, A.: Gene transfer into human leukemia cell lines by electroporation: experience with exponentially decaying and square wave pulse. *Leuk. Res.*, **15**, 507–513 (1991).

13. **Luo, D. and Saltzman, M. W.:** Enhancement of transfection by physical concentration of DNA at the cell surface. *Nat. Biotechnol.*, **18**, 893–895 (2000).
14. **Geller, A. L. and Breakfield, X. O.:** A defective HSV-1 vector expresses *Escherichia coli*  $\beta$ -galactosidase in cultured peripheral neurons. *Science*, **241**, 1667–1669 (1988).
15. **Wang, S. and Vos, J. M. H.:** An HSV/EBV based vector for high efficient gene transfer to human cells *in vitro/in vivo*. *J. Virol.*, **70**, 8422–8430 (1996).
16. **Banerjee, S., Livanos, L., and Vos, J. M. H.:** Therapeutic gene delivery in human B-lymphoblastoid cells by engineered non-transforming Epstein-Barr virus. *Nat. Med.*, **1**, 1303–1308 (1995).
17. **Wang, F., Xianping, L., Annis, B., and Faustmen, D. L.:** Tap-1 and Tap-2 gene therapy selectively restores conformationally dependent HLA class 1 expression in Type 1 diabetic cells. *Hum. Gen. Ther.*, **6**, 1005–1017 (1995).
18. **Ikeno, M., Inagaki, H., Nagata, K., Morita, M., Ichinose, H., and Okazaki, T.:** Generation of human artificial chromosomes expressing naturally controlled guanosine triphosphate cyclohydrolase 1 gene. *Genes Cells*, **7**, 1021–1032 (2002).