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Calcium enhanced delivery of tetraarginine-PEG-lipid-coated DNA/protamine complexes

T. Fujita[a], M. Furuhata[a], Y. Hattori[a], H. Kawakami[b], K. Toma[b], Y. Maitani[a,*]

[a] Institute of Medicinal Chemistry, Hoshi University, Ebara 2-4-41, Shinagawa-ku, Tokyo 142-8501, Japan
[b] The Noguchi Institute, Kaga 1-8-1, Itabashi-ku, Tokyo 173-0003, Japan

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ABSTRACT

As we have previously reported the delivery of plasmid DNA (DNA) complexed with oligoarginine-PEG artificial lipids (oligoarginine/DNA complexes), we focused on tetra- and decaarginine (Arg4, Arg10) to improve transfection efficiency by both the formation of oligoarginine-coated DNA complexed with protamine (PD), and the addition of Ca^{2+} after formation of complexes. The efficiency of DNA condensation was determined by gel electrophoresis. Cellular uptake and transfection efficiency were evaluated in human cervical carcinoma HeLa cells using flow cytometry and luciferase assay. Oligoarginine-coated PD enhanced transfection efficiency significantly more than complexes where Arg10 in both vectors exhibited higher transfection efficiency than Arg4. As assessed by gel retardation assay, high gene expression by Arg10 may be explained by Arg4 binding DNA more strongly than Arg10. The addition of Ca^{2+} to incubation medium increased transfection efficiency of Arg4-coated PD 70-fold, similar to that of Arg10-coated PD alone without an increase of cellular uptake, suggesting that Ca^{2+} induced the release of DNA from complexes in endosomes. Only Arg4 with low cytotoxicity could gain an advantage from Ca^{2+} in transfection, but Arg10 with relatively high cytotoxicity could not. The present results demonstrate that Arg4-coated PD with Ca^{2+} has great potential as an efficient non-viral vector with low toxicity.

1. Introduction

As a new gene therapy, cationic lipid-mediated gene transfer is well established and widely used (Felgner et al., 1987; Song et al., 1998; Cotten et al., 1990; Kircheis et al., 1999; Kakizawa et al., 2001). Several cell-penetrating peptides (CPPs), less than 30 amino acid residues in length, such as HIV-1 Tat fragments, have attracted much attention. CPPs contain a number of basic amino acid residues, and can deliver their associated molecules into cells (Derossi et al., 1994; Oehlke et al., 1998; Vives et al., 1997; Pooga et al., 1998; Futaki et al., 2001b; Morris et al., 2001). Oligoarginine has similar characteristic to CPPs (Mitchell et al., 2000; Wender et al., 2000; Futaki et al., 2001a,b). Oligoarginine-modified vectors with the ability to cross the plasma membrane are anticipated for the efficient delivery of plasmid DNA (DNA). Accordingly, oligoarginine incorporated into nanoparticles and liposomes was reported (Kogure et al., 2004; Zhang et al., 2006). Investigations delineating the influence of arginine length on the transfection efficiency and uptake of oligoarginine have reported that oligoarginine of eight residues in length showed higher transfection efficiency than arginine residues of 4–16 (Futaki et al., 2001a), and internalization efficiency was observed to depend on the chain length of oligoarginine peptides (Nakase et al., 2004). Octaarginine-linked stearyl residue (stearyl-Arg8) showed high transfection efficiency of DNA (Futaki et al., 2001a; Kogure et al., 2004), and efficient delivery of small interfering RNA (siRNA) (Tonges et al., 2006).

We previously reported that oligoarginine (Arg_n; $n = 4$, 6, 8, 10) conjugated 3,5-bis(dodecyloxy)benzamide (BDB) lipids with a poly(ethylene glycol) (PEG) spacer as a novel gene vector ((Arg)*n*-B). The transfection efficiency and cellular uptake of (Arg)*n*-B/DNA complexes ((Arg)*n*-B/D) increased as the number of arginine residues increased (Furuhata et al., 2006a). In contrast, with oligoarginine-modified liposome, tetraarginine-modified liposome alone showed the highest cellular uptake, and was able to deliver associated smaller protein highly among oligoarginine lipids (Furuhata et al., 2006b). We therefore hypothesized that the low transfection efficiency of Arg4-B was due to an inability to release DNA in cytoplasm. It has been reported that polycations like protamine neutralize DNA and facilitate release of DNA from cationic lipid vectors (Read et al., 2003) and therefore DNA condensed using protamine sulfate (PD), was complexed with liposomes (Li et al., 1998). Also calcium ion enhances transfection efficiency to facilitate the transfer of large molecules from endosome compartment to the cell cytoplasm (Palmer et al., 2003; Sandhu et al., 2005; Shiraishi et

* Corresponding author. Tel.: +81 3 5498 5048; fax: +81 3 5498 5048.
E-mail address: yoshie@hoshi.ac.jp (Y. Maitani).

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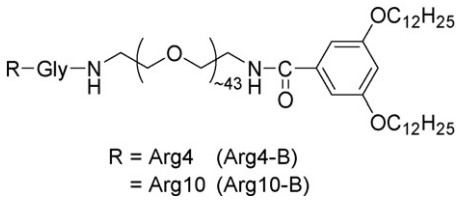

Fig. 1. Chemical structure of (Arg)*n*-B.

al., 2005). Here we used (Arg)*n*-B instead of liposomes to complex with PD and added calcium ion into the incubation medium after formation of complex to release DNA from vectors. Since (Arg)*n*-B forms micelles in water (Furuhata et al., 2008), by using the same method for post-PEGylation of liposomes with PEG lipid, PD could be coated by (Arg)*n*-B ((Arg)*n*-B-PD) as a novel vector. Furthermore, we recently found that at the concentration below critical micelle concentration (CMC), Arg10-B/DNA complexes (Arg10-B/D) exhibited significantly higher gene expression than that above CMC, 25 μM (Furuhata et al., 2008). Hence here we decreased from 25 μM to 5 μM for (Arg)*n*-B vectors.

In this study, we focused on (Arg)*n*-B (*n* = 4, 10) to improve vectors (i) by decreasing the concentration (Arg)*n*-B/D, (ii) by forming (Arg)*n*-B-coated PD, and (iii) by the addition of calcium ion into the medium. We demonstrate that the addition of Ca^{2+} to the medium dramatically increased the transfection efficiency of Arg4-B-PD with lower cytotoxicity.

2. Materials and methods

2.1. Materials

(Arg)*n*-B (Fig. 1) was synthesized as previously reported (Furuhata et al., 2006a). Protamine sulfate (grade III) and chloroquine were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Pica gene luciferase assay kit was purchased from Toyo Ink (Tokyo, Japan). Bicinchoninic acid (BCA) protein assay reagent was obtained from Pierce (Rockford, IL, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and Lipofectamine™ 2000 were purchased from Invitrogen Corp. (Carlsbad, CA, USA). All other chemicals used were of reagent grade.

The DNA encoding the luciferase gene under the control of the CMV promoter (pCMV-luc) was constructed as previously described (Igarashi et al., 2006). The plasmid pEGFP-C1 encoding the enhanced green fluorescent protein (EGFP) under the CMV promoter was purchased from Clontech (Palo Alto, CA, USA). Protein-free preparations of pCMV-luc and pEGFP-C1 were purified following alkaline lysis using maxiprep columns (Qiagen, Hilden, Germany). FITC-labeled 20-mer randomized oligodeoxynucleotide (FITC-ODN) was synthesized with a phosphodiester backbone (Sigma Genosys Japan, Hokkaido, Japan).

2.2. Preparation of (Arg)n-B/D and (Arg)n-B-PD complexes

Two kinds of vector/DNA complexes were prepared, (Arg)*n*-B/D and (Arg)*n*-B-PD. To prepare (Arg)*n*-B/D and (Arg)*n*-B/FITC-ODN, (Arg)*n*-B aqueous solution was added to an aqueous solution of DNA (pCMV-luc, pEGFP-C1 or FITC-ODN) where (Arg)*n*-B was fixed at a charge ratio (±) of 1.0, with gentle shaking. Here, one molecule of Arg4- and Arg10-B was considered as (+1) charge, not depending on the number of arginine residues.

To prepare (Arg)*n*-B-PD, anionic PD at a charge ratio (±) of 0.3 was prepared by the addition of an aqueous solution of protamine to the DNA aqueous solution with rapid vortexing (2 μg protamine per 2 μg DNA). Then, the (Arg)*n*-B aqueous solution was added to anionic PD suspension at a charge ratio (±) of (Arg)*n*-B to DNA of 1.0. Each complex was left at room temperature for 10–15 min.

2.3. Electrophoretic mobility and particle size and zeta potential determination

The degree of condensation afforded by oligoarginine was investigated using agarose gel retardation. (Arg)*n*-B/D was loaded onto 1.5% agarose gel, and DNA bands were visualized by ethidium bromide staining. Particle size distributions and zeta potentials of vector/DNA complexes were measured 10–15 min after formation by the dynamic and electrophoresis light scattering method, respectively (ELS-Z2, Otsuka Electronics Co. Ltd., Osaka, Japan) at 25 °C, after dilution to an appropriate volume with Milli-Q water.

2.4. Gene transfection

Human cervical carcinoma HeLa cells were kindly provided by Toyobo Co., Ltd. (Osaka, Japan) and grown in DMEM supplemented with 10% FBS at 37 °C in a humidified 5% CO_2 atmosphere. HeLa cell cultures were prepared by plating cells in a 35 mm culture dish 24 h prior to each experiment. The cells were washed three times with 1 ml of serum-free DMEM. For transfection, each vector, complexed with 2 μg of DNA or FITC-labeled ODN per well, was diluted with serum-free DMEM to 1 ml, and then gently applied to the cells.

For gene expression, after vector/DNA complexes were incubated with cells for 3 h at 37 °C in serum-free DMEM, 1 ml of DMEM containing 10% FBS was added to the cells, which were incubated further for 21 h. For transfection with Lipofectamine™ 2000 (LA2000), 5 μL of LA2000 was used for 2 μg of DNA to form a DNA complex in Opti-MEM, according to the manufacturer's protocol. The incubation condition was the same as stated above. Gene transfer efficiency was measured in triplicate.

For the treatment with chloroquine, after cells were incubated for 10 min with serum-free DMEM of 1 ml with 100 μM chloroquine, gene transfection was carried out; particle vectors with DNA were incubated with cells for 4 h at 37 °C in serum-free DMEM with 100 μM chloroquine, the medium with chloroquine was replaced with 1 ml of DMEM containing 10% FBS, and then the cells were incubated for further 20 h. Twenty four hours following the gene transfection, luciferase expression was measured as described above.

For Ca^{2+} supplementation studies, vector/DNA complexes were immediately added to tubes containing an aqueous solution of 1 M $CaCl_2$ at the desired concentration and left at room temperature for 30 min before diluting to the final volume with serum-free DMEM. After incubation for 3 h in serum-free DMEM (1 ml), the cells were further incubated for 21 h at 37 °C in DMEM (2 ml) containing 5% FBS. Ca^{2+} was in medium throughout the incubations.

2.5. Luciferase assay

In the transfection experiment, incubation was terminated by washing the plates three times with cold phosphate-buffered saline (pH 7.4, PBS) as previously reported (Furuhata et al., 2006a). Cell lysis solution was added to the cell monolayers and subjected to freezing at −80 °C and thawing at 37 °C, followed by centrifugation at 15,000 rpm for 5 s. Luciferase expression in the cells was measured according to the instructions accompanying the Pica gene luciferase assay system. The protein concentration of the supernatants was determined with BCA reagent using bovine serum albumin as a standard and cps/μg protein was calculated.

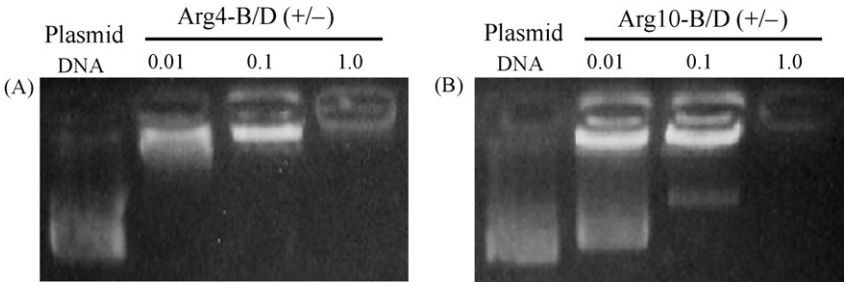

Fig. 2. Electrophoresis of Arg4-B/D (A) and Arg10-B/D (B) on agarose gels at charge ratios (±) of 0.01–1.0.

2.6. Flow cytometry

After gene transfection by incubation for 3 h at 37 °C in serum-free DMEM with or without 2 mM $CaCl_2$, the dishes were washed two times with 1 ml of PBS, and the cells were detached with 0.05% trypsin and 0.53 mM EDTA solution. The cells were centrifuged at 1500 × *g*, and the supernatant was discarded. The cells were resuspended with PBS containing 0.1% BSA and 1 mM EDTA, and directly introduced to a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 488 nm argon ion laser, as previously reported (Furuhata et al., 2006a).

2.7. Fluorescence microscopy

Cells were exposed for 3 h to the vector/DNA complexes in serum-free DMEM, and then cultured for another 21 h in DMEM containing 10% FBS. The cells were washed with PBS three times and then fixed with 4% paraformaldehyde. GFP expression was observed by fluorescence microscopy (ECLIPSE TS100-F, Nikon, Tokyo, Japan), and was imaged using the 465–495 nm excitation beam of a mercury lump. The contrast level and brightness of the images were adjusted.

2.8. Cell viability assay

Cell viabilities upon transfection using vector/DNA with Ca^{2+} were evaluated with a WST-8 assay (Dojindo, Kumamoto, Japan). Cells were seeded at a density of 3×10^4 cells/ml in growth medium containing serum per well in 96-well culture plates, and were transfected with complexes of 2 μg/ml DNA. After 24 h of incubation, the number of viable cells was determined by absorbance measured at 450 nm on an automated plate reader.

2.9. Data analysis

Significant differences in the mean values were evaluated by Student's unpaired *t*-test and one-way ANOVA followed by post-hoc analysis using Dunnett's test. A *p*-value of less than 0.05 was considered significant.

3. Results

3.1. Characteristics of vectors

To confirm the ability of (Arg)*n*-B to bind DNA, we prepared Arg4- and Arg10-B/D at various charge ratios and evaluated them by gel retardation assay. The results showed that Arg4-B at charge ratios (±) of more than 0.01, and Arg10-B at a charge ratio (±) of 0.1 completely bound DNA (Fig. 2). This finding suggested that Arg4-B interacted with DNA stronger than Arg10-B. In this experiment, therefore, the complexes were used at a charge ratio (±) of 1.0.

Particle sizes and zeta potential of the complexes, and Arg4- or Arg10-coated PD were about 150–230 nm and about +15–20 mV, respectively. (Arg)*n*-B is water-soluble, and hence, could coat PD. It was confirmed that the zeta potential of (Arg)*n*-B-PD changed from negatively charged PD alone at a charge ratio (±) of 0.3 (about −30 mV) to a positive charge (Fujita et al., 2008).

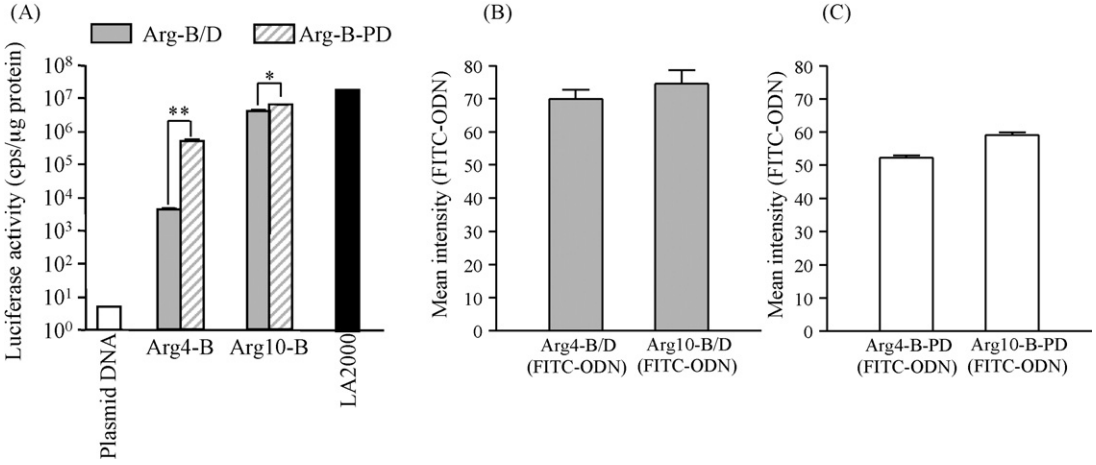

Fig. 3. Luciferase activity in HeLa cells after transfection using (Arg)*n*-B-PD at a charge ratio (±) of PD of 0.3 and at (Arg)*n*-B to DNA of 1.0 (A). Cellular uptake of (Arg)*n*-B/D (B) and (Arg)*n*-B-PD (C) using (Arg)*n*-B to 2 μg of FITC-ODN at a charge ratio (±) of 1.0. After incubation for 3 h in serum-free DMEM (1 ml), for luciferase activity, the cells were further incubated for 21 h at 37 °C in DMEM (2 ml) containing 10% FBS, and for FACS analysis, the cells were treated with trypsin. Each bar represents the mean ± S.D. of three experiments. $^*p < 0.05$ and $^{**}p < 0.01$.

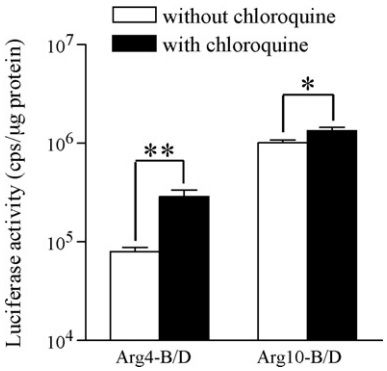

Fig. 4. Luciferase activity in HeLa cells after transfection using 5 μM (Arg)*n*-B/D complexes at a charge ratio (±) of 1.0 with or without chloroquine. After a 10 min incubation period with serum-free DMEM of 1 ml with 100 μM chloroquine, gene transfer was carried out. After incubation for 4 h at 37 °C in serum-free DMEM with 100 μM chloroquine, the medium with chloroquine was replaced with 1 ml of DMEM containing 10% FBS and the cells were incubated for a further 20 h.

3.2. Luciferase expression and cellular uptake of (Arg)n-B/D and (Arg)n-B-PD

Previous work has shown that below CMC (25 μM), 5 μM Arg10-B/D complexes exhibited significantly higher (1.5-fold) transfection activity than 25 μM (Furuhata et al., 2008). Hence, we used 5 μM (Arg)*n*-B when forming DNA complexes. Similar to the result at 25 μM (Furuhata et al., 2006a), 5 μM Arg10-B/D complexes exhibited higher luciferase activity than Arg4-B/D complexes (Fig. 3A). According to the result of gel retardation assay, low transfection efficiency by Arg4-B may be due to interaction with DNA and the inability to release.

In order to decrease the interaction of Arg4-B with DNA, we prepared negatively charged PD at a charge ratio (±) of 0.3 and then PD was coated with (Arg)*n*-B. Since PD exhibited the highest luciferase activity at a charge ratio (±) of 0.3 among 0.1–0.5 (Supplement Fig. 1), (Arg)*n*-B-PD was prepared at a charge ratio (±) of (Arg)*n*-B to DNA of 1.0, using PD at a charge ratio (±) of 0.3. Arg4- or Arg10-B-PD enhanced transfection efficiency significantly more than Arg4- or Arg10-B/D complexes, respectively, where Arg10-B-PD increased transfection efficiency up to commercial gene transfection reagent LA2000 ($p > 0.05$) (Fig. 3A). This finding also suggested that Arg4-B could not release DNA from complexes better than Arg10-B even when the negative charge of DNA was partially decreased by protamine.

To further examine the ability of (Arg)*n*-B vectors to carry genes into cells, we assayed cell internalization using FITC-ODN by flow cytometry. Unlike the large differences of transfection, similar uptake was observed by oligoarginine lengths in (Arg)*n*-B/D or (Arg)*n*-B-PD (Fig. 3B and C). These findings indicated that the

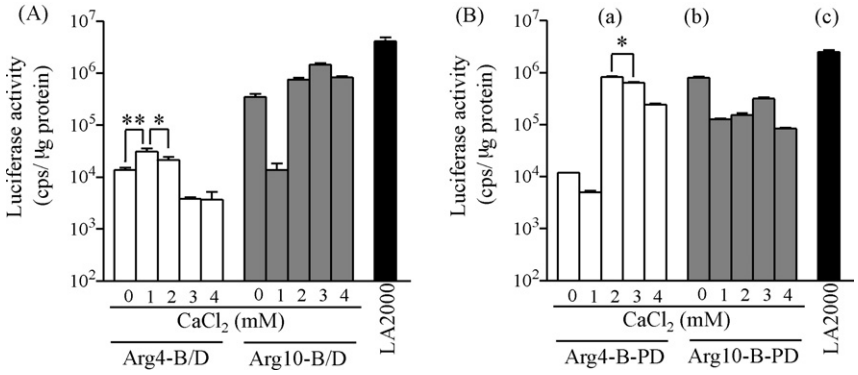

Fig. 5. Effect of Ca^{2+} concentration on luciferase activity of (Arg)*n*-B/D (A) and (Arg)*n*-B-PD (B). Vector/DNA complexes were left at room temperature for 30 min with serum-free DMEM containing an aqueous solution of $CaCl_2$, incubated with cells for 3 h, and further incubated for 21 h in DMEM containing 10% FBS. Each bar represents the mean ± S.D. of three experiments. Statistical analysis for comparing various Ca^{2+} concentrations with the control was performed using one-way ANOVA followed by post-hoc analysis using Dunnett's test. $^*p < 0.05$ and $^{**}p < 0.01$.

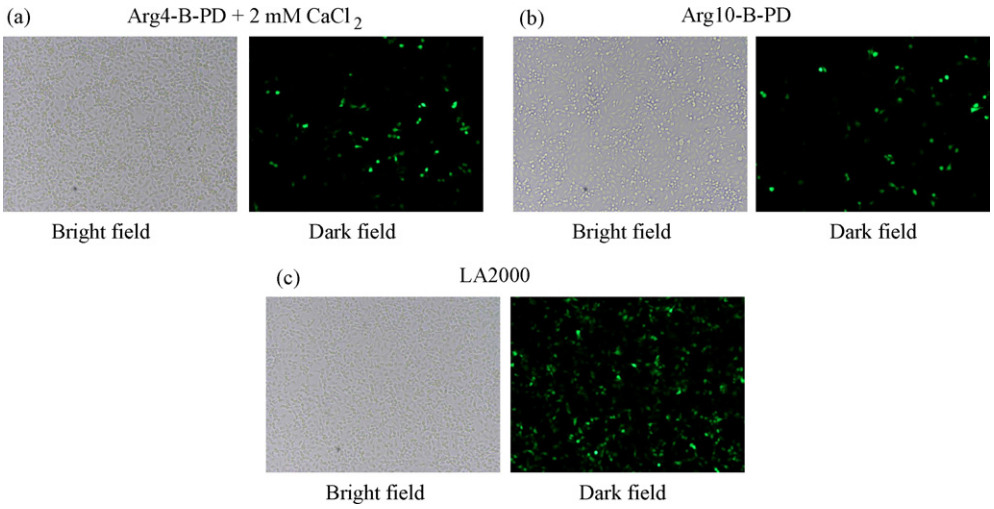

Fig. 6. GFP expression of Arg4-B-PD with Ca^{2+}, Arg10-B-PD and LA2000/DNA complexes by fluorescence microscopy (magnification ×100); (a), (b) and (c) correspond to the vector in Fig. 5(B).

uptake of vector/DNA complexes did not reflect their transfection activity.

3.3. Effect of chloroquine and Ca^{2+} on luciferase expression

Next, to clarify the discrepancy between transfection efficiency and cellular uptake, we investigated the endosomolytical properties of (Arg)*n*-B/D. Luciferase activities with chloroquine treatment prior to gene transfer were evaluated in the cells (Fig. 4). Chloroquine (100 μM) is known to avoid intracellular degradation by lysosomal enzymes (Carlisle et al., 2004; Hardy et al., 2006; Pelisek et al., 2006). The presence of chloroquine significantly improved the transfection efficiency of Arg4-B/D more than that of Arg10-B/D ($p < 0.01$).

Ca^{2+} is known to facilitate the transfer of large molecules from the endosome compartment to the cytoplasm (Palmer et al., 2003; Sandhu et al., 2005; Shiraishi et al., 2005). To release DNA from the complexes, we prepared DNA complexes in the presence of Ca^{2+} (Fig. 5). For (Arg)*n*-B/D, the transfection efficiency of Arg4 increased with Ca^{2+} concentration at 1 mM. Further increase in Ca^{2+} decreased transfection (Fig. 5A). The transfection efficiency of Arg10 increased with Ca^{2+}, except 1 mM Ca^{2+} (Fig. 5A).

For (Arg)*n*-B-PD, the transfection efficiency of Arg4 was significantly enhanced, ~70-fold more than that without Ca^{2+}, using 2 mM Ca^{2+} in the formulation (Fig. 5B). Any further increase in Ca^{2+} reduced transfection efficiency. The transfection efficiency of Arg10 decreased with the increase of Ca^{2+}. Arg4-B-PD with 2 mM Ca^{2+} (a) and Arg10-B-PD alone (b) showed highest transfection efficiency, similar to LA2000 (c) ($p > 0.05$). Ca^{2+} increased strongly transfection of Arg4-B-PD more than Arg4-B/D, but not that of Arg10-B-PD.

To examine the distribution of gene expression, we observed the gene expression of the plasmid pEGFP-C1 with (Arg)*n*-B-PD using fluorescence microscopy (Fig. 6). A slightly lower level of GFP protein was observed in cells treated with Arg4-B-PD with 2 mM Ca^{2+} and Arg10-B-PD alone than with LA2000, corresponding to the results of luciferase activity, shown in Fig. 5B (a), (b) and (c).

3.4. Effect of Ca^{2+} on cellular uptake of (Arg)n-B-PD and cytotoxicity

To investigate the decreased transfection efficiency of Arg10-B-PD with Ca^{2+}, cellular uptake was measured. We added Ca^{2+} to medium after formation of complex because it was reported that this procedure enhanced transfection (Lam and Cullis, 2000). Surprisingly, 2 mM Ca^{2+} decreased the cellular uptake of both Arg4- and Arg10-B-PD (Fig. 7). This was likely due to increase of size of (Arg)*n*-B-PD and decrease binding sites on the surface of cells.

It is well known that Ca^{2+} can be toxic to cells. The toxicities of (Arg)*n*-B-PD were assayed by determining cell viability following 24 h exposure of (Arg)*n*-B-PD corresponding to 0.2 μg DNA and a total lipid dose of approximately 5 μM of (Arg)*n*-B-PD per well. As shown in Fig. 8, Arg4-B-PD in the absence and presence of 1–4 mM Ca^{2+} exhibited little toxicity, whereas Arg10-B-PD alone and in the presence of 1 mM Ca^{2+} and LA2000/DNA complexes were highly toxic where cell viability was only ~57% with Arg10-B-PD alone and ~40% with LA2000/DNA complex. On the other hand, 77–100% cells were viable with Arg4-B-PD in the presence of up to 2 mM Ca^{2+}. This finding suggested that Arg10-B-PD with high toxicity did not receive a Ca^{2+} effect on increased transfection efficiency.

4. Discussion

This study demonstrates that we dramatically improved transfection efficiency by forming (Arg)*n*-B-coated PD, and that

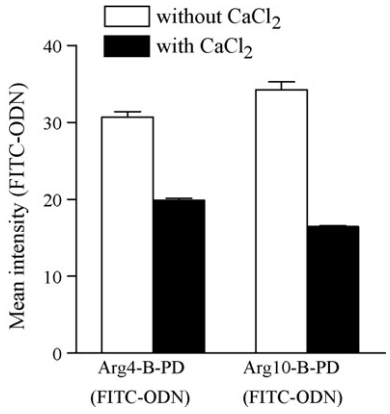

Fig. 7. Cellular uptake of Arg4-B-PD and Arg10-B-PD. (Arg)*n*-B at a charge ratio (±) of 1.0 was mixed with 2 μg of FITC-ODN. Cells were incubated for 3 h in serum-free DMEM with 2 mM Ca^{2+} and treated with trypsin before FACS analysis. Each bar represents the mean ± S.D. of three experiments.

of Arg4-B-PD by the addition of calcium ion into incubation medium.

The results demonstrate that (Arg)*n*-B could coat PD. It is well known that LPD vectors are non-viral vehicles for gene delivery composed of polycation-condensed DNA complexed with liposomes. Here we used protamine as a polycation. Protamine has been reported to enhance transfection efficiency by the ability of nuclear localization signaling (Sorgi et al., 1997; Ni et al., 1999). It was also reported that cationic PEG lipid can be inserted into preformed liposomes (Palmer et al., 2003). In this study, protamine-condensed DNA was covered with cationic oligoarginine-PEG lipids, a novel vector. PD type vector showed higher transfection efficiency than the complex type; Arg4- and Arg10-B-PD enhanced transfection efficiency 130-fold and 1.5-fold more than Arg4- and Arg10-B/D complexes, respectively. After (Arg)*n*-B-PD penetrated the plasma membrane, (Arg)*n*-B-PD might more easily release DNA from complexes than (Arg)*n*-B/D, likely due to the negative charge of DNA being partially decreased by protamine because (Arg)*n*-B interacted strongly with DNA.

Improvements of transfection did not arise from improved uptake into cells. In this regard, a number of studies have indicated that the cationic lipids contained in lipoplexes plays a direct role in stimulating uptake into cells, being due to the positive charge on lipoplexes (Miller et al., 1998). In this study at 5 μM as well as at

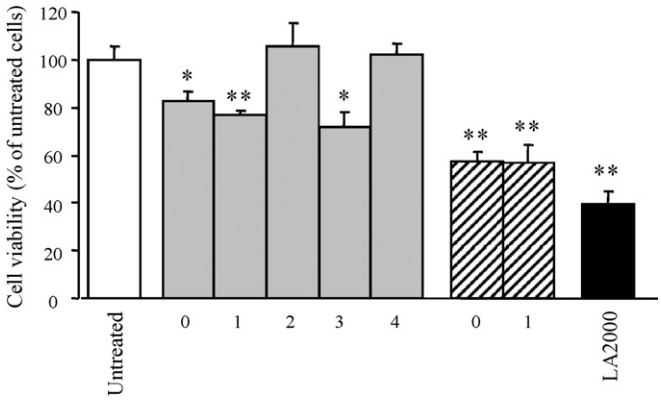

Fig. 8. Toxicity of Arg4-B-PD, Arg10-B-PD and LA2000/DNA 24 h after transfection was assessed by cell viability. Gray and hatched bars represent Arg4-B-PD and Arg10-B-PD, respectively. The numbers refer to the concentration (mM) of $CaCl_2$. Each bar represents the mean ± S.E. of four experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the untreated cells.

25 μM (Furuhata et al., 2006a), Arg10-B/D exhibited about 1000-fold higher transfection efficiency to Arg4-B/D in spite of similar cellular uptake (Fig. 3B).

Arg10 with both complex- and PD-type vectors exhibited higher transfection efficiency than Arg4 without an increase of cellular uptake. Similar uptake was observed with Arg4- and Arg10-B/ODN in our study. Nakase et al. (2004) reported that the cellular uptake of hexadecaarginine peptide was higher than octaarginine and tetraarginine peptide, and that of tetraarginine peptide was slight in living cells. The discrepancy with this report might be due to the different observations by using labeled oligoarginine (Nakase et al., 2004) and cargo, labeled ODN associated with oligoarginine lipid.

Presumably, since both Arg4- and Arg10-B have the same structure except for oligoarginine length, the small difference of DNA binding affinity may be caused by oligoarginine length. The poor transfection efficiency of Arg4-B/D may be due to the process of releasing DNA from the endosome compartment to the cell cytoplasm and/or the penetration of DNA into the nucleus because cellular uptake by Arg4- and Arg10-B/D was not significantly different. Ca^{2+} and chloroquine in the increase of transfection efficiency in Arg4-B was much higher than in Arg10-B. Chloroquine (100 μM) is known to avoid intracellular degradation by lysosomal enzymes (Carlisle et al., 2004; Hardy et al., 2006; Pelisek et al., 2006), which is an ion-transporting ATPase inhibitor that disrupts endosomes by preventing their acidication, enhanced CPP release from macropinosomes (Shiraishi et al., 2005). Ca^{2+} enhanced transfection of DNA/cationic lipid particulate complex by the increase of cellular uptake of DNA/lipid complex and facilitated the transfer of DNA from the endosome compartment to the cytoplasm (Palmer et al., 2003; Sandhu et al., 2005). In this study, Ca^{2+} enhanced the transfection of (Arg)*n*-B/D without increasing its cellular uptake. Ca^{2+} stimulated the release of DNA from endosoma/lysosomal compartments as well as the lysosomotrophic agent chloroquine. This was supported by the findings that Arg4 bound with DNA stronger than Arg10 by gel retardation assay. The difference of transfection efficiency by oligoarginine length may be caused from the different interaction with DNA.

Ca^{2+} could enhance Arg4-B-mediated gene transfer, but not Arg10-B-PD. This finding corresponded with the observation that efficient transfection of cationic liposome in the presence of Ca^{2+} is observed at lower cationic lipid-to-DNA charge ratios than in the absence of Ca^{2+} (Lam and Cullis, 2000) when lower cationic lipid was considered Arg4-B. Ca^{2+} might substitute to some extent for Arg4-B in the complex and interact directly with DNA in the complex. This finding was possibly explained that Arg10-B had more cytotoxicity than Arg4-B, which may be caused by longer Arg residues. Therefore, Arg10-B could not gain an advantage from Ca^{2+} because Ca^{2+} also induced cytotoxicity. The presence of Ca^{2+} results in a maximum increase in Arg4-B-PD transfection potency of about 70-fold and this increase was derived from the ability of Ca^{2+} to assist in destabilizing the endosomal membrane following uptake, not from an increase of uptake (Palmer et al., 2003). These findings provide new insights into the mechanism of how oligoarginine enters cells.

In summary, we improved oligoarginine vectors by the formation of (Arg)*n*-B-PD. Furthermore, the addition of Ca^{2+} to incubation medium increased the transfection efficiency of Arg4-B-PD, but not that of Arg10-B-PD. This difference may be due to Arg4-B binding DNA strongly and it might be hard to release the gene in cytoplasm compared with Arg10-B. Also, since Arg10-B exhibited relatively higher cytotoxicity than Arg4-B, it could not gain an advantage from Ca^{2+}. We found that the combination of Ca^{2+} and protamine could enhance Arg4-B-mediated gene transfer. Arg4-B-PD with Ca^{2+} has potential as an efficient non-viral vector with low toxicity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2008.09.060.

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