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Design, synthesis and gene delivery efficiency of novel oligo-arginine-linked PEG-lipids: Effect of oligo-arginine length

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Abstract

The design, synthesis, and evaluation of in vitro gene delivery efficacy of a novel series of oligo-Arg-lipid conjugates are described. 3,5-Bis(dodecyloxy)benzamide (BDB) was employed as the lipid component, and a poly(ethylene glycol) (PEG) spacer was introduced between the C-terminal of oligo-Arg and the amide group of BDB. Four derivatives with various oligo-Arg lengths (ArgN-PEG-BDB; $N = 4$, 6, 8, 10: the number of arginine residues) were prepared, and the effect of oligo-Arg length on the gene transfection was investigated in HeLa cells. Transfection efficiency increased as the number of arginine residues increased. Arg10-PEG-BDB showed the highest transfection efficiency, without severe toxicity to cells. These findings well corresponded to the cellular association of the Arg-PEG-BDB/DNA complex determined by flow cytometry. Even in the presence of serum, Arg10-PEG-BDB achieved appreciable cellular association and attained high gene expression. Thus, Arg10-PEG-BDB is potentially a simple and useful gene delivery tool, because one need only to mix it with plasmid DNA and apply the complexes to the cells even in a serum-containing medium.

Keywords: Cell penetrating peptides; Oligo-arginine; Gene delivery

1. Introduction

Cationic lipid-based gene transfection constitutes one of the most promising alternatives to the use of viral vectors (Felgner et al., 1987; Song et al., 1998; Cotten et al., 1990; Kircheis et al., 1999; Brown et al., 2000). However, the low-level transfection efficiency compared with viral vectors is considered a major limitation in the application to gene therapy. The poor efficiency is supposed to arise from the endocytic route of internalization of cationic lipids complexed with DNA. Therefore, novel and more efficient synthetic vectors, hopefully with a different cell internalization mechanism, are desired.

Recently, a cellular internalization method using short peptides derived from protein-transduction domains has attracted much attention. Several cell penetrating peptides (CPPs), such as HIV-1 Tat fragments, less than 30 amino acid residues in length, have the capability of crossing a plasma membrane (Derossi et al., 1994; Vives et al., 1997; Oehlke et al., 1998; Pooga et al., 1998; Futaki et al., 2001a; Morris et al., 2001). In addition, they can deliver their associated molecules into cells. The Tat peptide has been reported to be capable of delivering β-galactosidase (120 kDa) to various organs when administered intraperitoneally to mice (Schwarze et al., 1999), and even nanoparticles (Lewin et al., 2000) and liposomes (Torchilin et al., 2001) can be delivered into cells. Although the mechanism of cell internalization is still incompletely understood, it is reported to be different from that of liposome vectors, which are internalized via an energy-independent pathway (Derossi et al., 1994; Vives et al., 1997). Oligo-arginine (Arg) conjugates were demonstrated to have characteristics similar to CPPs in cell translocation (Mitchell et al., 2000; Wender et al., 2000; Futaki et al., 2001a).

Although investigations delineating the influence of Arg length on the transfection efficiency and uptake of oligo-Args have been reported (Mitchell et al., 2000; Wender et al., 2000; Futaki et al., 2001a,b), there is no report about oligo-Arg-linked poly(ethylene glycol) (PEG) lipids alone as a gene vector. The

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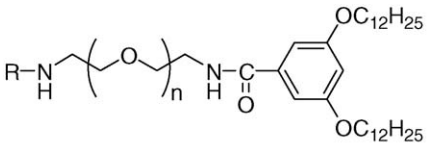
aim of this study was to design and synthesize simple and effective vectors for use in gene delivery. Oligo-Arg-linked PEG-lipids might have the ability to electrostatically stabilize naked DNA and mediate gene transfection through a non-endocytotic pathway.

In the present study, we synthesized oligo-Arg-lipids of quite different structure from a reported one (Futaki et al., 2001b), employing 3,5-bis (dodecyloxy)benzamide (BDB) as the lipid component, and introducing a PEG spacer between the C-terminal of oligo-Arg and the amide group of BDB (oligo-Arg-PEG-BDB). Four derivatives with various oligo-Arg lengths were prepared, and the effect of oligo-Arg length on the gene delivery efficacy was investigated in HeLa cells. We demonstrate that the arginine 10-mer exhibits the highest transfection efficiency in HeLa cells among our series of compounds.



1 R = H
2 R = Fmoc-Arg(Pmc)
3 R = Arg(Pmc)
4 R = Fmoc-[Arg(Pmc)]$_2$
5 R = [Arg(Pmc)]$_2$
6 R = Fmoc-[Arg(Pmc)]$_3$
7 R = [Arg(Pmc)]$_3$
8 R = Fmoc-[Arg(Pmc)]$_4$
9 R = Arg$_4$ (Arg4-PEG-BDB)
10 R = Fmoc-[Arg(Pbf)]$_6$
11 R = Arg$_6$ (Arg6-PEG-BDB)
12 R = Fmoc-[Arg(Pbf)]$_6$[Arg(Pmc)]$_2$
13 R = Arg$_8$ (Arg8-PEG-BDB)
14 R = [Arg(Pmc)]$_4$
15 R = Fmoc-[Arg(Pbf)]$_6$[Arg(Pmc)]$_4$
16 R = Arg$_{10}$ (Arg10-PEG-BDB)

Fig. 1. Chemical structures of ArgN-PEG-BDBs and their synthetic intermediates.

2. Materials and methods

2.1. Materials

All amino acid derivatives and coupling reagents were obtained from Kokusan chemical Co., LTD (Tokyo, Japan). PEG 2000 was obtained from Kanto Kagaku Co., Ltd (Tokyo, Japan), and converted into its diamino derivative using a reported procedure (Slama and Rando, 1980). 9-fluorenylmethyl-oxycarbonyl-[Arg(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)]$_6$-OH (Fmoc-[Arg(Pbf)]$_6$-OH) was purchased from Peptide Institute, Inc (Osaka, Japan). The Pica gene luciferase assay kit was purchased from Toyo Ink (Tokyo, Japan). Bicinchonic acid (BCA) protein assay reagent and EZ-Label Fluorescein Protein Labeling Kit were obtained from Pierce (Rockford, IL, USA). Lipofectamine^{TM} 2000, fluorescein isothiocyanate (FITC)-transferrin and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen Corp. (Carlsbad, CA, USA). FITC-Tat was purchased from AnaSpec, Inc. (San Jose, CA, USA). All other chemicals used were of reagent grade. Fetal bovine serum (FBS) was purchased from Life Technologies (Grand Island, NY, USA). In the following section, the number of moles of PEG in compounds is calculated by taking the molecular weight of the PEG fragment as 2000.

2.2. Synthesis of oligo-Arg-PEG-BDBs

3,5-Bis(dodecyloxy)benzoic acid (Balagurusamy et al., 1997) (1 g) and benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) (0.38 g) were dissolved in *N*,*N*-dimethylformamide (DMF) (30 mL), and the solution was stirred at room temperature for 1 h. Diamino poly(ethylene glycol) 2000 (5 g) was added to the solution, and the reaction was carried out overnight. The resulting mixture was poured into water, and extracted with $CHCl_3$. The organic layer was washed with water, dried over $MgSO_4$, and filtered, and the organic solvent was evaporated under reduced pressure. The residue was purified by silica gel column chromatography to give **1** (Fig. 1). Fmoc-Arg(2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc))-OH (1.07 g) and PyBOP (0.84 g) were dissolved in DMF (20 mL), and the solution was stirred at room temperature for 1 h. **1** (2 g) was added to the solution, and the reaction was carried out overnight. The resulting mixture was poured into water, and extracted with $CHCl_3$. The organic layer was washed with water, dried over $MgSO_4$, filtered, and the organic solvent was evaporated under reduced pressure. The residue was purified by Sephadex LH-20 to give **2**. To a solution of **2** (2.45 g) in CH_2Cl_2, piperidine was added, and the solution was stirred at room temperature for 30 min. The resulting mixture was directly purified using Sephadex LH-20 to give **3**. Similarly, **4**, **5**, **6**, **7** and **8** were synthesized step by step. A solution of **8** (0.2 g) in trifluoroacetic acid (TFA)/water (9/1, 2 mL) was stirred at room temperature for 3 h, and concentrated in vacuo. The residue was dissolved in CH_2Cl_2. Piperidine was added to the solution, and the reaction carried out at room temperature for 30 min. The resulting mixture was directly purified by silica gel column chromatography to give Arg4-PEG-BDB (**9**). MALDI-TOF MS (α-CHCA) 3096.13, 3140.03, 3184.71 ($[M+H]^+$). Similar to the synthesis of **2** and **8, 10** and Arg6-PEG-BDB (**11**), respectively, were synthesized from Fmoc-[Arg(Pbf)]$_6$-OH and **1**. MALDI-TOF MS (α-CHCA) 3584.62, 3628.17, 3672.29 ($[M+H]^+$). Similar to the synthesis of **2** and **8, 12** and Arg8-PEG-BDB (**13**), respectively, were synthesized from Fmoc-[Arg(Pbf)]$_6$-OH and **5**. MALDI-TOF MS (α-CHCA) 3670.02, 3714.17, 3757.87 ($[M+H]^+$). Similar to the deprotection of the Fmoc group of **2** and **8** gave **14**. Similar to the synthesis of **2** and **8, 15** and Arg10-PEG-BDB (**16**), respectively, were synthesized from Fmoc-[Arg(Pbf)]$_6$-OH and **14**. MALDI-TOF MS (α-CHCA) 4123.36, 4166.18, 4209.92 ($[M+H]^+$).

2.3. Plasmid DNA and FITC-labeled oligodeoxynucleotide

The plasmid DNA (about 6740 bp) encoding the luciferase gene under the control of the CMV promoter (pCMV-luc) was supplied by Dr. Tanaka of the Mt. Sinai School of Medicine (NY, USA). The plasmid pEGFP-C1 encoding the green fluorescent protein (GFP) 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). The

FITC-labeled 20-mer randomized oligodeoxynucleotide (FITC-labeled ODN) was synthesized with a phosphodiester backbone (Sigma Genosys Japan, Hokkaido, Japan).

2.4. Preparation of FITC-labeled Arg10-PEG-BDB

FITC-labeled Arg10-PEG-BDB was prepared by applying an EZ-Label Fluorescein Protein Labeling Kit to Arg10-PEG-BDB.

2.5. Cell culture

Human cervical carcinoma cells (HeLa) were kindly provided by Toyobo Co., Ltd. (Osaka, Japan). HeLa cells were grown in DMEM supplemented with 10% FBS at 37 °C in a humidified 5% CO_2 atmosphere.

2.6. Gene transfection

An aqueous solution of plasmid DNA (pCMV-luc or pEGFP-C1) or FITC-labeled ODN was added to the oligo-Arg-PEG-BDB aqueous solution with gentle shaking to form oligo-Arg-PEG-BDB/DNA complexes. Each complex was left at room temperature for 10–15 min. HeLa cell cultures were prepared by plating cells in a 35-mm culture dish 24 h prior to each experiment. The cells were washed 3 times with 1 mL of serum-free DMEM. For transfection, each oligo-Arg-PEG-BDB/DNA complex (2 μg of plasmid DNA and 100 μg of oligo-Arg-PEG-BDB per well were fixed with a charge ratio (+/−) of oligo-Arg to plasmid DNA of 4.25–5.5 (Arg4-PEG-BDB-Arg10-PEG-BDB)) was diluted with serum-free DMEM to 1 mL, then gently applied to the cells. Two sets of conditions were employed: (a) after incubation for 3 h at 37 °C in serum-free DMEM, DMEM (1 mL) containing 10% FBS was added, and the cells were further incubated for 21 h, (b) incubation for 24 h at 37 °C in DMEM (2 mL) containing 10% FBS. For transfection with Lipofectamine™ 2000, 5 μL of Lipofectamine™ 2000 was used for 2 μg of the plasmid DNA to form a DNA complex in Opti-MEM according to the manufacturer's protocol. The incubation conditions were the same as stated above. The measurement of gene transfer efficiency was performed in triplicate.

2.7. Inhibition of endocytosis

Arg10-PEG-BDB (100 μg) containing 20% FITC-labeled Arg10-PEG-BDB or its complex with plasmid DNA (2 μg), FITC-transferrin and FITC-Tat were diluted with DMEM containing 10% FBS to 1 mL and then incubated with cells for 3 h at either 4 °C or 37 °C.

2.8. Luciferase assay

Luciferase expression was measured according to the instructions accompanying the luciferase assay system. Incubation was terminated by washing the plates three times with cold phosphate buffered saline (pH 7.4) (PBS). Cell lysis solution (Pica gene) 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. The supernatants were frozen and stored at −80 °C until the assays. Aliquots of 20 μL of the supernatants were mixed with 100 μL of luciferase assay system (Pica gene) and counts per second (cps) were measured with a chemoluminometer (Wallac ARVO SX 1420 multilabel counter, Perkin-Elmer Life Science, Japan, Co. Ltd., Kanagawa, Japan). The protein concentration of the supernatants was determined with BCA reagent using bovine serum albumin as a standard and cps/μg protein was calculated.

2.9. Flow cytometry

At the end of the incubation, the dishes were washed two times with 1 mL of PBS, and the cells were detached with 0.05% trypsin and 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. Data for 10,000 fluorescent events were obtained by recording forward scatter (FSC) and side scatter (SSC) with green (530/30 nm) fluorescence.

2.10. Confocal microscopy

GFP expression in HeLa cells was observed after the gene transfection with incubation for 3 h at 37 °C in serum-free DMEM. DMEM (1 mL) containing 10% FBS was added as described above. After the medium was removed, the cells were washed with PBS and fixed with 10% formaldehyde PBS at room temperature for 20 min, and washed three times with PBS. Then, the cells were coated with Aqua Poly/Mount (Poly science, Warrington, PA, USA) to prevent fading and covered with coverslips. The fixed cells were observed with a Radiance 2100 confocal laser scanning microscope (BioRad, CA, USA). GFP was imaged using the 488-nm excitation beam of an argon laser, and fluorescence emission was observed with a filter HQ515/30. The contrast level and brightness of the images were adjusted.

2.11. Particle size determination

The oligo-Arg-PEG-BDB/DNA complex and Lipofectamine™ 2000/DNA complex were formed as described in Gene Transfection. Particle size was measured by the dynamic light-scattering method (ELS-800, Otsuka Electronics Co. Ltd, Osaka, Japan) at 25 °C after diluting the Arg-PEG-BDB/DNA complex, the Lipofectamine™ 2000/DNA complex, Lipofectamine™ 2000 and Arg10-PEG-BDB to an appropriate volume with Milli-Q water.

2.12. Cytotoxicity

HeLa cells were seeded at a density of 1×10^4 cells per well in 96-well plates and maintained for 24 h before transfection in DMEM supplemented with 10% FBS. The cells were washed

with serum-free DMEM. The culture medium was replaced with serum-free DMEM (50 μL) including various concentrations of Arg10-PEG-BDB ranging from 2.5 to 1000 μM, or the DNA complex as described in Section 2.6. After incubation for 3 h at 37 °C with serum-free DMEM (50 μL), DMEM (50 μL) containing 10% FBS was added. The cells were further incubated for 21 h. The number of surviving cells was determined by a WST-8 assay (Dojindo Laboratories, Kumamoto, Japan). Cell viability was expressed as the ratio of the A_{450} of cells treated with the DNA complex to that of the control samples.

2.13. Data analysis

Significant differences in the mean values were evaluated by student's unpaired *t*-test. A *p*-value of less than 0.05 was considered significant.

3. Results

3.1. Luciferase expression of Arg-PEG-BDB/DNA complexes

We prepared four oligo-Arg-linked lipids of various lengths, Arg4-PEG-BDB, Arg6-PEG-BDB, Arg8-PEG-BDB and Arg10-PEG-BDB (Fig. 1). We evaluated the transfection efficiency of oligo-Arg-PEG-BDB by assaying luciferase activity. HeLa cells were transfected with oligo-Arg-PEG-BDB complexed with pCMV-luc. Transfection was conducted for 3 h in the absence or presence of serum, and the cells were cultured for another 21 h in the presence of serum. According to the result of a preliminary experiment, the oligo-Arg-PEG-BDB concentration appeared to be an important factor in obtaining a high transfection efficiency. All oligo-Arg-PEG-BDB derivatives exhibited luciferase activity at 40 μg/mL with a charge ratio of cation to plasmid of 2.2–1.7 (Arg4-PEG-BDB-Arg10-PEG-BDB). As the concentration of oligo-Arg-PEG-BDB increased, the level of luciferase activity also increased. However, it was observed that Arg8-PEG-BDB became cytotoxic at 200 μg/mL in the gene transfection. Therefore, the concentration of oligo-Arg-PEG-BDB should be restricted. A concentration of 100 μg/mL, corresponding to 32, 29, 27 and 25 μM for Arg4-PEG-BDB, Arg6-PEG-BDB, Arg8-PEG-BDB and Arg10-PEG-BDB, respectively, was used in the subsequent experiments.

Fig. 2(A) and (B) demonstrates that the longer oligo-Arg showed stronger luciferase activity irrespective of the serum in the medium. Arg10-PEG-BDB showed the highest level of activity among the oligo-Arg-PEG-BDB derivatives, with about 40-fold, 11-fold and 4-fold higher transfection efficiencies than Arg4-PEG-BDB, Arg6-PEG-BDB and Arg8-PEG-BDB, respectively, on 3 h-incubation in serum-free medium (Fig. 2(A)). Arg10-PEG-BDB showed about 1/5 the transfection efficiency of Lipofectamine™ 2000, a commercial gene transfection reagent, even in a serum-containing medium (Fig. 2(B)). Serum tended to decrease the gene transfection efficiency of oligo-Arg-PEG-BDB, an exception being Arg10-PEG-BDB.

3.2. Cellular uptake of Arg-PEG-BDB/DNA complexes

To confirm the ability of oligo-Arg-PEG-BDB to carry genes into cells, we prepared ODN labeled with FITC and assayed the cell internalization of the Arg8-PEG-BDB, Arg10-PEG-BDB/ODN or Lipofectamine™ 2000 complex by flow cytometry (Fig. 3). Cells were exposed for 3 h to the FITC-labeled ODN complex in the absence (Fig. 3(A)) or presence of serum (Fig. 3(B)), cultured for another 21 h in the presence of serum, and then trypsinized. A flow cytometric analysis demonstrated that cell internalization occurred in each case and in the absence of serum, Lipofectamine™ 2000 showed the strongest labeling

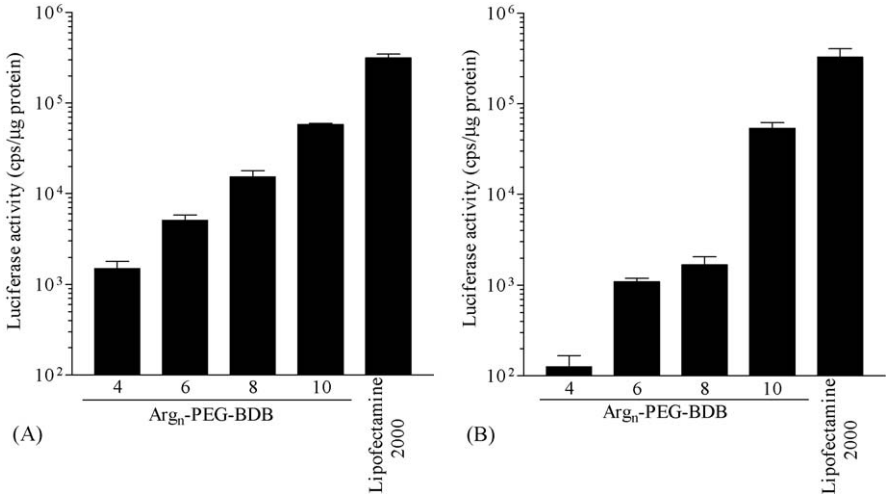


Fig. 2. In vitro luciferase activity after transfection of HeLa cells using oligo-Arg-PEG-BDB/DNA complexes. The complexes were prepared by mixing 2 μg of pCMV-luc with 100 μg of oligo-Arg-PEG-BDB or Lipofectamine™ 2000 (5 μL). The charge ratio of cation to plasmid was 4.25–5.5 (Arg4-PEG-BDB-Arg10-PEG-BDB). (A) After incubation for 3 h at 37 °C in serum-free DMEM, DMEM (1 mL) containing 10% FBS was added, and the cells were further incubated for 21 h. (B) Cells were incubated for 24 h at 37 °C in DMEM (2 mL) containing 10% FBS. Each bar represents the mean ± S.D. of three experiments.

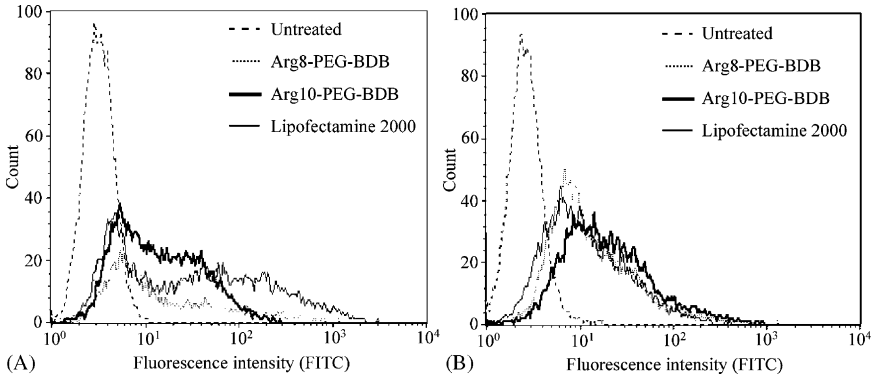


Fig. 3. Cellular uptake of the DNA complexes of Arg8-PEG-BDB and Arg10-PEG-BDB. Arg8-PEG-BDB, Arg10-PEG-BDB (100 μg) or Lipofectamine™ 2000 was mixed with 2 μg of FITC-labeled ODN. (A) The cells were incubated for 3 h in serum-free DMEM and incubated another 21 h in DMEM containing 10% FBS. (B) The cells were incubated for 24 h in DMEM (2 mL) containing 10% FBS and treated with trypsin before FACS analysis. Rough dotted line, untreated; Subtle dotted line, Arg8-PEG-BDB; Bold line, Arg10-PEG-BDB; Plain line, Lipofectamine™ 2000.

intensity among vectors. However, in the presence of serum, the intensity of the signal was greater in the cells transfected with Arg10-PEG-BDB than those with Lipofectamine™ 2000 and Arg8-PEG-BDB, indicating that Arg10-PEG-BDB could carry more DNA into the cells than Lipofectamine™ 2000 and Arg8-PEG-BDB. It suggests that the uptake efficiency of Arg10-PEG-BDB was not susceptible to the serum. These results suggest that the optimal number of oligo-Arg was 10 among the tested. Therefore, Arg10-PEG-BDB was used in subsequent experiments.

3.3. GFP gene transfection

To examine the distribution of transfection in cells, we observed the transfection efficiency of Arg10-PEG-BDB with the plasmid pEGFP-C1 using confocal microscopy. Cells were exposed for 3 h to the Arg10-PEG-BDB or Lipofectamine™ 2000/DNA complex in the absence of serum, and then cultured for another 21 h in the presence of serum. Next, the cells were fixed with 10% paraformaldehyde and visualized by confocal microscopy (Fig. 4). A slightly lower level of GFP protein was observed in the cells treated with Arg10-PEG-BDB than with Lipofectamine™ 2000, corresponding to the results of luciferase expression (Fig. 2). Similar results were obtained in a flow cytometric study for Arg10-PEG-BDB and Lipofectamine™ 2000 in serum-free incubation (Fig. 3(A)).

3.4. Effect of low temperature on the uptake

To confirm the internalization mechanism of our CPP, we prepared FITC-labeled Arg10-PEG-BDB and examined the effect of temperature on the cellular uptake of complexes. In order to avoid changes in the cell internalization character, we only incorporated FITC-labeled Arg10-PEG-BDB (20%). The endocytosis marker transferrin (Tf) and another CPP of Tat were used as control. The cells were exposed to FITC-labeled Arg10-PEG-BDB or its DNA complex, FITC-transferrin and FITC-Tat for 3 h at either 4 °C or 37 °C in the presence of serum. Then, the cells were trypsinized and analyzed by flow cytometry (Fig. 5). FITC-labeled Arg10-PEG-BDB and its DNA complex showed about an 86% lower internalization efficiency at 4 °C than at 37 °C. No significant difference in the mean fluorescence was observed between the DNA complex and FITC-labeled Arg10-PEG-BDB alone at 4 and 37 °C (Fig. 5(C)). This finding suggests that the internalization by our oligo-Arg-PEG-BDB and its DNA complexes was considerably inhibited at low temperature.

3.5. Particle size

Particle sizes of Arg10-PEG-BDB, Lipofectamine™ 2000, Arg10-PEG-BDB/DNA and Lipofectamine™ 2000/DNA were estimated using dynamic light scattering in Milli-Q water 15–60 min after the complex had formed. Particles of

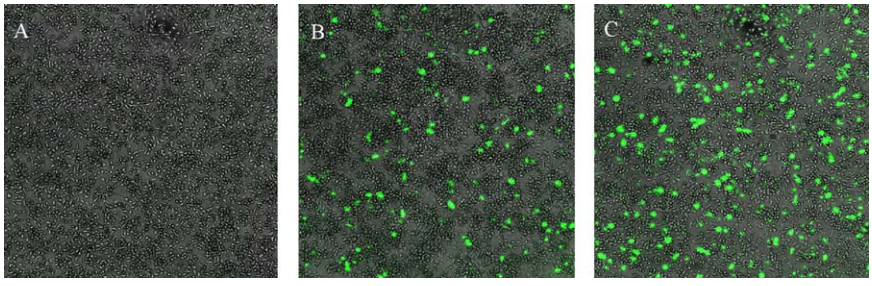


Fig. 4. Analysis of GFP expression by confocal microscopy. The DNA complexes were prepared by mixing 2 μg of pEGFP with Arg10-PEG-BDB (100 μg) or Lipofectamine™ 2000 (5 μL). The cells were incubated for 3 h in serum-free DMEM and incubated another 21 h in DMEM (1 mL) containing 10% FBS before confocal microscopy. Panel A, untreated; panel B, Arg10-PEG-BDB; panel C, Lipofectamine™ 2000. All views were recorded with the same camera acquisition parameters.

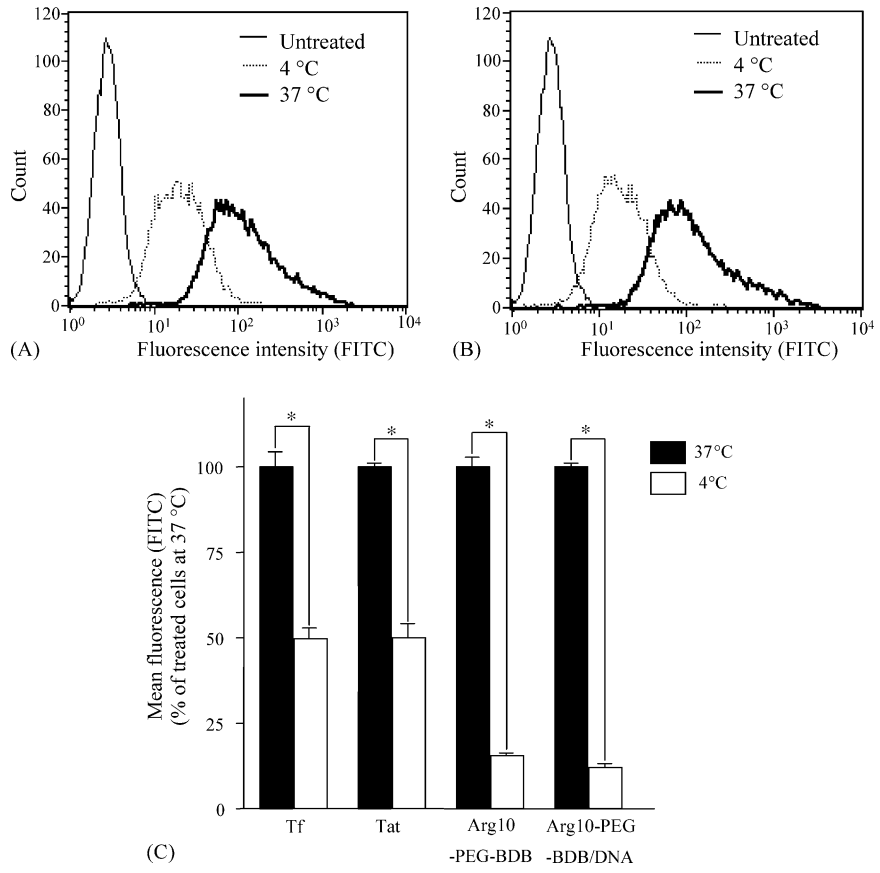


Fig. 5. Effect of temperature on the cellular uptake of FITC-labeled Arg10-PEG-BDB and its DNA complexes. (A) Arg10-PEG-BDB containing 20% FITC-labeled Arg-PEG-BDB. (B) The complex of plasmid DNA with FITC-labeled Arg10-PEG-BDB. Arg10-PEG-BDB was labeled with fluorescein isothiocyanate at the N-terminal of oligo-Arg as described under experimental procedures. The DNA complexes were prepared by mixing 2 μg of plasmid DNA with FITC-labeled Arg10-PEG-BDB (100 μg). The cells were incubated for 3 h at 4 or 37 °C in DMEM (1 mL) containing 10% FBS, and treated with trypsin before flow cytometry. (C) The mean fluorescence intensity of FITC-labeled Arg10-PEG-BDB, its DNA complexes, FITC-transferrin (Tf) (25 μg/mL) and FITC-Tat (Tat) (10 μM) compared with treated cells at 37 and 4 °C. Closed bar, 37 °C; Open bar, 4 °C. Each bar represents the mean ± S.D. of three experiments. $p < 0.05$ are marked by asterisk.

Arg10-PEG-BDB and the Arg10-PEG-BDB/DNA complex were about 300 and 1000 nm, respectively, suggesting that Arg10-PEG-BDB formed micelles. Those of Lipofectamine™ 2000 and the Lipofectamine™ 2000/DNA complex were about 400 nm and about 800 nm, respectively.

3.6. Cytotoxicity

The cytotoxicity of the vectors was assessed by the WST-8 assay using cells incubated with Arg10-PEG-BDB in serum-free medium for 3 h and in a serum-containing medium for another 21 h. The IC_{50} value was about 550 μM for Arg10-PEG-BDB (data not shown). The cytotoxicity of the Arg10-PEG-BDB/DNA complex was almost equal to that of the Lipofectamine™ 2000/DNA complex in both sets of conditions (Fig. 6).

4. Discussion

A peptide consisting of oligo-Arg has been shown to be translocated through cell membranes as efficiently as other CPPs (Mitchell et al., 2000; Wender et al., 2000; Futaki et al., 2001a). In these cases, the oligo-Arg length and the hydrophobic moiety of oligo-Arg conjugates were important factors for the uptake and transfection in cells (Futaki et al., 2001b). The aims of this study were to design and synthesize novel oligo-Arg-linked PEG-lipids, and determine the optimal length of oligo-Arg for transfection efficiency and to develop an effective gene delivery vector.

We prepared oligo-Arg-modified lipids with a PEG linker. The transfection experiment using pCMV-luc showed the ability of oligo-Arg-PEG-BDB to carry genes into cells. The transfection efficiency of the longer oligo-Arg was higher. The transfection efficiency in the absence of serum increased about four times as two arginine residues were added. The highest level of luciferase activity in cells was observed in Arg10-PEG-BDB irrespective of serum, suggesting that the optimal number of arginine residues for transfection was 10.

A limitation of the transfection assay with pCMV-luc is that it does not provide any information on the percentage of cells transfected. Therefore, we used pEGFP-C1 as another plasmid DNA. Arg10-PEG-BDB showed a similar efficiency in gene transfer to pCMV-luc, and a slightly lower fluorescence to Lipofectamine™ 2000, on confocal microscopy and flow cytometry in serum-free medium. The transfection assay with pEGFP-C1 and Arg10-PEG-BDB provided evidence that the

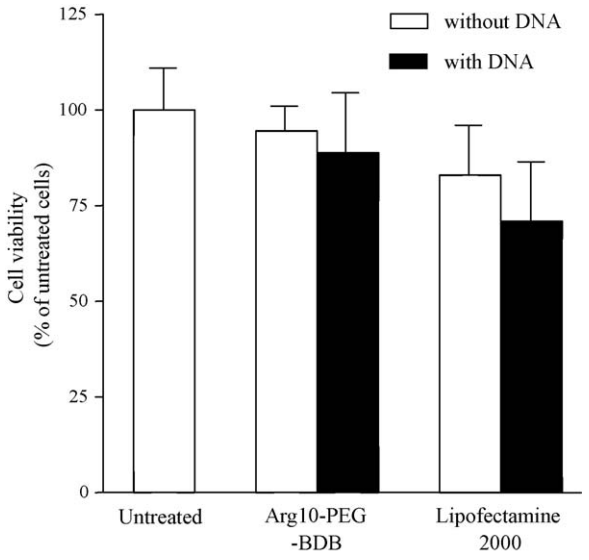


Fig. 6. Cytotoxicity of Arg10-PEG-BDB and Lipofectamine™ 2000 complexed with the plasmid DNA. The cytotoxicity on transfection was evaluated using the WST-8 assay. HeLa cells were seeded at a density of 1×10^4 cells per well in 96-well plates and maintained for 24 h before the gene transfection in DMEM containing 10% FBS. The culture medium was replaced with serum-free DMEM (50 μL) containing DNA (2 μg) complexed with Arg10-PEG-BDB (100 μg) or with Lipofectamine™ 2000 (5 μL). After incubation for 3 h at 37 °C in DMEM without FBS, DMEM (50 μL) containing 10% FBS was added. The cells were further incubated for another 21 h. Open bars, cell viability in the absence of plasmid DNA; Closed bars, cell viability in the presence of plasmid DNA. Each bar represents the mean ± S.D. of three experiments.

observed transfection efficiency resulted from low overall levels of gene insertion in many cells, not from a low overall percentage of cells transfected with a few cells receiving large numbers of the reporter gene.

We also showed that both Arg8-PEG-BDB and Arg10-PEG-BDB were able to carry plasmid DNA inside the cells by flow cytometry. To removing the surface bound Arg-PEG-BDB/DNA complexes, we washed the cells with PBS and treated them with trypsin (Richard et al., 2003). In the presence of serum, Arg10-PEG-BDB was able to deliver more DNA into the cells than Lipofectamine™ 2000 and Arg8-PEG-BDB, but this finding did not reflect in transfection efficiency. It was suggested that the process of cellular uptake and/or transfection might be different between Arg10-PEG-BDB and Lipofectamine™ 2000. No severe cytotoxicity was observed during 24 h incubation at 25 μM (100 μg/mL) of Arg10-PEG-BDB (Fig. 6). Notably, even in the presence of serum, the Arg10-PEG-BDB/DNA complex achieved appreciable cellular association to attain a high level of gene expression.

Mitchell et al. reported that 15 arginine residues were internalized significantly more effectively than 20 arginine residues (Mitchell et al., 2000). Wender et al. reported that nine arginine residues, the maximum number used in their experiment, were superior to shorter oligomers in terms of cellular uptake as determined by flow cytometry (Wender et al., 2000). They also demonstrated that the presence of at least six arginine residues is important for cellular uptake (Wender et al., 2000). Using oligomers composed of 4–16 arginine residues, Futaki et al. demonstrated that there was an optimal number of arginine residues (Arg8) for cellular internalization by microscopic observation (Futaki et al., 2001a). They also reported that stearylation of Arg8 at the N-terminal (stearyl-Arg8) improved the transfection efficiency compared with Arg8 alone, giving the highest transfection efficiency from stearyl-Arg4 to stearyl-Arg16 at a charge ratio of cation to DNA of 2:1 (Futaki et al., 2001b). In our case, Arg10-PEG-BDB showed a higher transfection efficiency and cellular uptake than Arg8-PEG-BDB.

Therefore, effect of the length of oligo-Arg on transfection efficiency has to be taken into consideration. In transfection experiments with the oligo-Arg-PEG-BDB/DNA complex, the arginine residues may be partly used for translocation through the plasma membrane and partly for the formation of complex with plasmid DNA. The longer oligo-Arg would be needed for the intracellular delivery of oligo-Arg-PEG-BDB/DNA than the uptake of oligo-Arg-PEG-BDB alone. In this respect, oligo-Arg-PEG-BDB with more than ten arginine residues would be more effective for transfection if no cytotoxicity is observed. Cytotoxicity may be influenced by incubation with or without serum and/or cell type. The cellular uptake and transfection efficiency of oligo-Arg-PEG-BDB/DNA complexes were proportional to the chain length of oligo-Arg. This finding suggests that the limiting factor of gene transfection was the uptake of complex to the plasma membrane, and not the release of DNA from the endosome compartment to the cell cytoplasm or the penetration of DNA into the nucleus.

It is interesting that two quite different designs of oligo-Arg-lipids, Arg10-PEG-BDB (25 μM) (Fig. 2) and stearyl-Arg8 (30 μM) (Futaki et al., 2001b), showed comparable transfection efficiencies to Lipofectamine™ 2000. The presence of a PEG linker and the lipid structure does not seem to affect the length of oligo-Arg suitable for the transfection and uptake. The transfection efficiency is likely to be influenced more by the length of oligo-Arg than by overall structural features such as the anchor lipids, linker groups, and direction of oligo-Arg relative to the lipid portion, etc.

The cellular translocation by CPP was initially proposed to be an energy-independent process. Most papers report no difference in uptake between 37 and 4 °C (Vives et al., 1997; Futaki et al., 2001a). However, more recent papers suggest that the majority of the translocation occurs via an energy-dependent pathway and that the translocation of CPP is reduced by endocytosis inhibitors (Fischer et al., 2002; Vives, 2003; Drin et al., 2003). To investigate the internalization mechanism of our system, we constructed FITC-labeled Arg10-PEG-BDB and its DNA complex, and examined the temperature-dependence of their internalization using flow cytometry. Therefore, the internalization mechanism of our system may have less of a contribution from energy-independent processes. FITC-labeled Arg10-PEG-BDB alone showed a similar internalization efficiency to the FITC-labeled Arg10-PEG-BDB/DNA complex at 37 °C for 3 h, suggesting that Arg10-PEG-BDB and Arg10-PEG-BDB/DNA follow a similar pathway. These findings conflict with the report that the quantitative uptake of free CPP or CPP coupled to cargo can differ (Fischer et al., 2004), but corresponds to the report that the cellular entry of both stearyl-Arg8 and the stearyl-Arg8/DNA

complex occurs mainly through endocytosis (Khalil et al., 2004).

Given the particle size of Arg10-PEG-BDB, aggregates such as micelles would be formed since PEG-lipid conjugates were reported to form micelles (Lukyanov et al., 2002). One explanation for the transfection efficiency of Arg10-PEG-BDB could be that Arg10-PEG-BDB aggregates and behaves similarly to polycationic micelles (Itaka et al., 2003).

5. Conclusions

In summary, we synthesized oligo-Arg containing lipids with a PEG spacer as novel gene vectors, and found that their transfection efficiency increased as the number of arginine residues increased. Among them, Arg10-PEG-BDB showed the highest transfection efficiency in HeLa cells. Arg10-PEG-BDB and its DNA complex may be internalized via energy-dependent processes.

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