Polyarginine Molecular Weight Determines Transfection Efficiency of Calcium Condensed Complexes

Nabil A. Alhakamy¹ and Cory J. Berkland¹,²,‡
¹Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS, USA 66047
²Department of Chemical & Petroleum Engineering, University of Kansas, Lawrence, KS, USA 66047

Abstract

Cell penetrating peptides (CPPs) have been extensively studied in polyelectrolyte complexes as a means to enhance the transfection efficiency of plasmid DNA (pDNA). Increasing the molecular weight of CPPs often enhances gene expression, but poses a risk of increased cytotoxicity and immunogenicity compared to low molecular weight CCPs. Conversely, low molecular weight CPPs typically have low transfection efficiency due to large complex size. Complexes made using low molecular weight CPPs were found to be condensed to a small size by adding calcium. In this study, complexes of low molecular weight polyarginine and pDNA were condensed with calcium. These complexes showed high transfection efficiency and low cytotoxicity in A549 carcinomic human alveolar basal epithelial cells. The relationship between transfection efficiency and polyarginine size (5, 7, 9 or 11 amino acids), polyarginine/pDNA charge ratios, and calcium concentrations were studied. Polyarginine 7 was significantly more effective than other polyarginines under most formulation conditions suggesting a link between cell penetration ability and transfection efficiency.

Keywords
plasmid DNA; polyarginine; cell-penetrating peptides; A549 cells; non-viral gene delivery; calcium chloride; transfection

1. INTRODUCTION

Many studies have shown that nucleic acids complex electrostatically with polycations to form polyplexes. Unfortunately, the most effective polycations are often the most toxic. Polyethylenimine (PEI), polylysine and similar polycations can mediate high levels of gene expression, but high molecular weight polycations are often required to effectively condense the DNA to small particles. The needed increase in molecular weight; however, often increases cytotoxicity. Cytotoxicity of polycations may be minimized by different approaches such as conjugation with hydrophilic or degradable polymers (e.g. hydroxypropyl methacrylamide (HPMA) or polyethylene glycol (PEG)). A simple

‡Corresponding Author: University of Kansas, Department of Pharmaceutical Chemistry, 2030 Becker Drive, Lawrence, Kansas 66047. Phone: (785) 864-1455. Fax: (785) 864-1454. berkland@ku.edu.
alterative is to identify ways to form small polyplexes using less cytotoxic polycations such as cell penetrating peptides (CPPs).\textsuperscript{1, 17–19}

CPPs are short sequences of amino acids characterized by their cationic or amphipathic nature that aid cellular uptake of different molecular cargo.\textsuperscript{1, 20–23} Two general methods have been examined for CPP-mediated gene delivery, which are chemical conjugation of CPPs with nucleic acids or complexation.\textsuperscript{11, 24–27} The negative charge of genetic material (e.g. DNA or siRNA) and the positive charge of CPPs can impede direct conjugation. On the other hand, genetic material can be electrostatically bound and neutralized when complexed with CPPs.\textsuperscript{1, 11, 28}

The most commonly known CPPs are human immunodeficiency virus-1 (HIV-1) transcriptional activator (TAT) protein, and the herpes simplex virus structural protein VP22. Sequence similarities between TAT, and VP22 indicated the importance of basic amino acids, such as arginine or lysine. Short peptides containing only arginine have the ability to translocate through cell membranes.\textsuperscript{1, 9, 24–33} Furthermore, polymers or CPPs with some of their residues altered with arginine or the guanidine group displayed highly enhanced transfection efficiency.\textsuperscript{32, 34}

The size, the charge, and the molecular weight of CPPs (e.g. polyarginine) play important roles in condensing DNA for delivering genetic material to target cells.\textsuperscript{35} CPPs containing cationic amino acids such as lysine or arginine were found to complex genetic material efficiently.\textsuperscript{36–38} CPPs rich with arginine also tended to possess high transfection efficiency.\textsuperscript{39–42} Often, polyarginine peptides delivered genetic material into cells more efficiently than other homopolymer peptides (e.g. polylysine and polyhistidine) of equal length. A study also determined the uptake of different polyarginine lengths (e.g. R3, R6, R9, R15 and R30) and found that simply increasing the length of polyarginine did not necessarily increase the uptake. Polyarginines between R7 and R20 maximized uptake by cells.\textsuperscript{41, 43, 44} Other groups studied the translocation of different polyarginine lengths (R4, R6, R8, R10, R12 and R16) and found that the R8 peptide maximized cellular uptake, while others found R6 to R9 translocated optimally through cell membrane. An analogous result occurred using polyarginines to mediate protein delivery.\textsuperscript{30, 41, 44} Thus, polyarginine size is essential for membrane permeability.

Some reports have suggested that low molecular weight polyarginine peptides are able to form complexes with DNA and promote transfection in different mammalian cell types.\textsuperscript{32, 45–47} Although reports suggest low molecular weight polyarginine/pDNA complexes may achieve modest transfection with negligible cytotoxicity, the large particle size (microparticles) is a limitation.\textsuperscript{32} Indeed, high molecular weight polycations often have the ability to condense DNA into small and stable complexes while low molecular weight polycations often yield large and unstable complexes.\textsuperscript{32, 47–49} A major shortcoming with high molecular weight polyarginines is cytotoxicity; however, low molecular weight polyarginines (< 10 kDa) typically exhibit poor transfection efficiency.

Calcium phosphate precipitation is a well-established approach to DNA condensation and gene delivery. Numerous \textit{in vitro} studies demonstrated that transfection efficiency of DNA
increased when formulated as a calcium phosphate-DNA co-precipitate. Other studies found that calcium chloride has a positive effect on the transfection efficiency of plasmid DNA (pDNA)-cationic liposome complexes.\textsuperscript{1, 50–53} Still other studies showed that calcium might increase the endocytosis rate or accelerate endosomal release before degradation by lysosomes.\textsuperscript{54, 55} While the effect of calcium on transfection is unclear, these studies encouraged exploration of calcium with complexes formed using CPPs, such as polyarginine. Consequently, calcium was found to condense these large complexes and increase the positive charge, which dramatically enhanced transfection.\textsuperscript{1, 11, 56} In fact, titrating calcium can directly affect complex size and stability. Also, released polyarginines may maintain CPP properties since they are not chemically conjugated using this approach.

Thus, the delivery of polyarginine/pDNA complexes condensed with calcium chloride may offer a safe non-viral gene delivery technique with potential for clinical application.\textsuperscript{1} This simple formulation (polyarginine/pDNA/Ca$^{2+}$) maintained the viability of A549 lung cancer epithelial cells while achieving high transfection efficiency. This method also yields a small and stable particle size, and is suitable for delivering large amounts of genetic material. In order to determine the importance of polyarginine molecular weight, \textit{in vitro} transfection efficiency studies were conducted using different polyarginines (R5, R7, R9, and R11) (Table 1), and different calcium chloride concentrations (0, 50, 100, 150, 300, and 600 mM).

## 2. MATERIALS AND METHODS

### 2.1. Materials

Plasmid DNA (pDNA) encoding firefly luciferase (pGL3, 4818 bp) was obtained from Promega (Madison, Wisconsin). The pDNA purity level was determined by UV-Spectroscopy and agarose gel electrophoresis. Polyarginine (Arg 5, 7, 9 and 11) were purchased from Biomatik (Cambridge, Ontario, Canada). Branched polyethylenimine (PEI, 25 kDa) was obtained from Sigma-Aldrich (Milwaukee, Wisconsin). A549 carcinoma human alveolar basal epithelial cell line was obtained from American Type Culture Collection (ATCC; Rockville, Maryland). The cell culture medium (F-12K Nutrient Mixture, Kaighn’s modified with L-glutamine) was purchased through Cellgro (Mediatech, Inc., Manassas, VA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Penicillin/Streptomycin was purchased from MB Biomedical, LLC (Solon, OH). Trypsin-EDTA was purchased from Invitrogen (Carlsbad, CA). Luciferase Assay System Freezer Pack and CellTiter 96® AQueous one solution cell proliferation assay (MTS) were obtained from Promega (Madison, Wisconsin). BCA Protein Assay Reagent (bicinchoninic acid) was purchased from Thermo Fisher Scientific Inc. Tris-acetate-EDTA (TAE) Buffer (10 x) was purchased from Promega (Madison, Wisconsin). Sterile water (DNase, RNase free) was purchased from Fisher Scientific. Calcium chloride dihydrate (CaCl$_2$. 2H$_2$O) was purchased from Fisher Scientific. Agarose (Medium-EEO/Protein Electrophorisis Grade) was purchased from Fisher Scientific. Bench Top DNA Ladder was purchased from Promega (Madison, WI). SYBR Green I Nucleic Acid Gel Stain was obtained from Invitrogen (Carlsbad, CA).
2.2. Preparation of Polyarginine/pDNA/Ca\(^{2+}\) Complexes

Polyarginine/pDNA complexes were prepared by adding 15 μL of polyarginine solution (different polymer nitrogen to pDNA phosphate (N/P) ratios) to 10 μL (0.1 μg/μL) of pDNA (TAE Buffer (1 x) was used as a solution for DNA storage), followed by fast pipetting for 20 second. At that point, 15 μL of identified molarity (e.g., 50, 300, and 600 mM) CaCl\(_2\) was added and mixed by fast pipetting. After preparing the complexes, they were stored at 4°C for 20–25 minutes.

2.3. Preparation of PEI/pDNA Complexes

PEI/pDNA complexes were prepared by adding 15 μL of PEI solution (N/P ratio 10) to 10 μL (0.1 μg/μL) of pDNA followed by fast pipetting for 20 seconds. After preparing the complexes, they were stored at 4°C for 20–25 minutes. Complexes were prepared immediately before each experiment.

2.4. Agarose Gel Electrophoresis

Complexes were prepared as defined previously and subsequently 4 μL of Tris-acetate-EDTA (TAE) buffer was added. Then, 4 μL of SYBR Green 1 was mixed with the complexes. Afterwards, the mixture was stored at 4°C for 20–25 minutes. After the storage, 7 μL of 6X DNA Loading Dye was added. A one kb DNA ladder was used. The mixture solutions were loaded onto a 1 % agarose gel, and electrophoresed for 30 minutes at 110 V.

2.5. Size and Zeta Potential

The particle size (effective diameter (nm)) of polyarginine/pDNA complexes with or without calcium chloride was determined by dynamic light scattering (Brookhaven Instruments, Holtsville, NY). The zeta potentials of the complexes were measured by Zeta PALS dynamic light scattering (Brookhaven Instrument, Holtsville, NY). All samples intended for particle size and zeta potential measurements were prepared using 10 mM Tris buffer, pH 7.4.

2.6. Cell Culture

A549 carcinomic human alveolar basal epithelial cell were grown in F-12K Nutrient Mixture media (Kaighn’s modified with L-glutamine) with 1% (v/v) Penicillin/Streptomycin and 10% (v/v) fetal bovine serum (FBS) at 37°C in 5% CO\(_2\) humidified air.

2.7. Transfection Studies

A549 cells were cultured in 96-well plates for 24 hr prior to transfection. The concentration of the cells in every well was approximately 80,000 cells/mL. The wells were washed once with serum-free media (SFM) and afterwards a 100 μL sample (which consisted of 20 μL of complex and 80 μL of SFM) was added to each well. Subsequently, a 96-well plate was incubated for 5 hr in an incubator. After 5 hr incubation, the sample was replaced with 100 μL of fresh serum medium and then incubated again for approximately 48 hr. In order to determine the gene expression of the complexes, the Luciferase Reporter Assay from Promega was used. The results of the transfections were expressed as Relative Light Units (RLU) per milligram (mg) of cellular protein and PEI/pDNA was used as a control. BCA
Protein Assay Reagent (bicinchoninic acid) was used to measure total cellular protein concentration in the cell extracts. The Luciferase Assay and BCA were measured by a microplate reader (SpectraMax; Molecular Devices Crope, CA).

### 2.8. Cytotoxicity Assay of Polyarginine

Cytotoxicity of polyarginines was determined using a CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) obtained from Promega (Madison, Wisconsin). A549 cells were cultured in a 96-well plate as described previously. After 24 h of incubation, the media were replaced with a sample consisting of 100 μL of fresh serum medium and 20 μL of MTS. Then, the plate was incubated for 3 hr in the incubator. In order to determine cell viability, the absorbance of each well was measured by a microplate reader (SpectraMax; Molecular Devices Crope, CA) at 490 nm and normalized to untreated control cells.

### 2.9. Optical microscopy

Cells were cultured, grown and incubated as mentioned above. Then, the complexes were added and cells were incubated again for 5 hr and 48 hr. Data were obtained using a Zeiss Axiovert 100 Microscope (Axiocam HRM).

### 2.10. Statistical Analysis

Data were analyzed by using GraphPad software. Statistical evaluation comparing the significance of the difference in gene expression (RLUs/mg protein) between the means of two data sets was performed using an unpaired t-test. One-way ANOVA, Tukey post test was used to analyze the differences when more than two data sets were compared.

### 3. RESULTS AND DISCUSSION

Agarose gel electrophoresis studies indicated that the complexes without calcium were robust enough to immobilize DNA when complexed with polyarginine 7, 9 and 11. DNA complexes with polyarginine 5 were less stable without calcium than with calcium (Figure 1). The ability of the four distinct molecular weights of polyarginine to form complexes with pDNA was also studied using agarose gel electrophoresis at N/P ratios of 5, 10, 20, 30, 40, and 60. Polyarginines 5, 7, 9 and 11 were complexed with pDNA at these different N/P ratios and then condensed with calcium chloride (300 mM) (Figure 2). The immobilization of pDNA indicated that different polyarginine peptides could form complexes with pDNA even with the lowest N/P ratio of 5.

An important characteristic of the polyarginine/pDNA/Ca\(^{2+}\) complex is the size. A broad study of the relationship between the calcium concentration (150, 300, and 600 mM) and the particle size was conducted. Figure 3 illustrates the particle sizes of polyarginine 5, 7, 9 and 11 complexed with pDNA as a function of calcium chloride concentration. The polyarginine/pDNA complexes generally showed a decrease in particle size as calcium concentration increased whereas the complexes without calcium showed a large particle size. The total positive charge of complexes also plays an important role in transfection efficiency and can increase the attractive force to the negative charge of the cell surface.
Overall, the zeta potential of polyarginine of 5, 7, 9 and 11/pDNA complexes increased considerably with increasing concentration of calcium chloride. For example, the zeta potential of polyarginine 7/pDNA complexes increased significantly from less than 5 to more than 20 mV with increasing concentration of calcium chloride (Figure 4). Clearly, PEI/pDNA complexes had the highest positive zeta potential. The lowest values were associated with polyarginine 5/pDNA complexes.

The cellular uptake of free polyarginine was previously reported to be proportional to the guanidine content.43, 57–60 Here, different polyarginines were complexed with pDNA and the transfection of A549 cells was studied as a function of polyarginine molecular weight, calcium concentration, and N/P ratio. Polyarginine/pDNA/Ca^{2+} complexes generally achieved high gene expression. Interestingly, there was no notable gene expression observed for the polyarginine complexes without calcium. Polyarginine 5 complexes had low gene expression with or without calcium. On the other hand, polyarginine 7, 9 and 11 complexes yielded high gene expression only when adding calcium chloride. Additionally, the transfection efficiency of the best polyarginine/pDNA/Ca^{2+} complexes was superior to PEI/pDNA complexes. Polyarginine 7 appeared significantly effective compared to the other polyarginines (Figure 5). Figure 6 compares the same data to illustrate the difference in transfection efficiency between the polyarginine peptides at difference N/P ratios, (CaCl_2 concentration 100 and 150 mM).

Calcium is an important component to condense the complexes, yielding small complexes with optimized DNA release.48 Consequently, only the combination of calcium and polyarginine effectively boosted gene transfection. In addition, others found that the cellular uptake of free polyarginine 16 was considerably higher than polyarginine 8, but the translocation into the cytosol was less efficient than polyarginine 8. In light of this, it is possible that the polyarginines near 8 amino acids may mediate uptake of the complexes and optimize cytosolic release.30 This may help explain why polyarginine 7 had the highest gene expression (RLUs/mg protein) compared to other polyarginines. Moreover, Figure 7 indicates that the transfection efficiency of pDNA/Ca^{2+} complexes without polyarginine peptides was significantly lower than the complexes with polyarginine when comparing the same calcium concentration.

One potential mechanism of the improved transfection may be the disintegration of these complexes as pH decreases (i.e. intracellular). Interestingly, data illustrated the particle size of the CPP/pDNA/Ca^{2+} complexes increased when the pH of media was decreased (pH 7 to 4) (Supplementary Figure 1). The particle counts (kilo counts per second; Kcps) for complexes of polyarginine 7/pDNA with calcium in acetate buffer media (pH 7, 5 and 4) (Supplementary Figure 2) decreased when pH decreased. These results suggested that calcium condensed CPP/pDNA complexes may swelled and dissociate as pH decreased, which is supported by previous reports of calcium/pDNA precipitates.61

Finally, an effective gene delivery vector must be able to deliver genetic material safely to target cells without cytotoxicity. Arginine is converted to ornithine by an enzyme called arginase and ornithine is the precursor of numerous polyamines, which are essential for cell proliferation.62 However, high molecular weight CPPs (e.g. polyarginine) can precipitate...
serum proteins and exhibit cytotoxicity at high concentration. Decreasing the molecular weight of polyarginine tends to decrease cytotoxicity. Here, a cytotoxicity assay was used to measure the cytotoxicity of free PEI and polyarginines (Figure 8). Polyarginine 5, 7, 9 and 11 showed little evidence of cytotoxicity against A549 cells even at very high concentrations (1 mg/mL). These results supported the promise that polyarginine/pDNA/Ca\(^{2+}\) complexes had high transfection efficiency while maintaining low cytotoxicity.

Micrographs of A549 cells showed that PEI/pDNA complexes had a negative effect on cell growth compared to polyarginine/pDNA/Ca\(^{2+}\) complexes (Figure 9). Images of the cells after 5 h and 48 h were similar when incubated with PEI/pDNA complexes suggesting inhibition of cell growth. On the other hand, the cells proliferated as expected when incubated with polyarginine 7/pDNA/Ca\(^{2+}\) complexes. Other reports suggest that PEI inhibits cell proliferation and alternatively, arginine may even have a slight enhancing effect. Additionally, we noticed that there was no precipitation when we used the low concentrations of CaCl\(_2\) (≤ 300 mM), but there was precipitation with the high concentrations of CaCl\(_2\) (> 300 mM). Micrographs were also supported by plots of total protein after 48 h of incubation (Figure 10). In general, polyarginine 5, 7, 9 and 11 had higher protein levels than PEI and were similar to control cell cultures. This finding suggests that PEI transfection was artificially amplified due to inhibition of cell proliferation.

4. CONCLUSIONS

Polyarginines have been successfully used as cell penetrating peptides for delivering large cargo such as genetic material. Polyarginine/pDNA complexes condensed with calcium chloride possessed excellent transfection efficiency compared to PEI complexes in A549 lung epithelial cells. Calcium chloride concentration played a significant role in controlling the complex size and charge, which were important for cell transfection. Moreover, calcium condensation of complexes allows the incorporation and release of low molecular weight polyarginine CPPs in the range of 7–11 amino acids, which may locally increase cell membrane permeability (e.g. within intracellular vesicles). Polyarginine 7 appeared especially effective compared to the other polyarginines. Polyarginine 5 was ineffective compared to the other polyarginines suggesting the minimum size should be > 5 amino acids. Furthermore, the low molecular weight polyarginine peptides (5, 7, 9 and 11) showed negligible cytotoxicity up to 5 mg/ml and maintained the expected cell proliferation rates. The incorporation of CPPs into polyelectrolyte complexes using this strategy potentially offers a safe and effective approach to gene delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References


Figure 1.
Agarose gels of polyarginine/pDNA complexes with and without CaCl$_2$ (300 mM) at N/P ratios of 5, 10, 20, 30, 40, and 60. (A) Polyarginine 5/pDNA complexes without CaCl$_2$ and (B) with CaCl$_2$. (C) Polyarginine 7/pDNA complexes without CaCl$_2$ and (D) with CaCl$_2$. (M) Refers to marker and (C) refers to control (pDNA).
Figure 2.
Agarose gels of polyarginine/pDNA complexes condensed with CaCl$_2$ (300 mM) at N/P ratios of 5, 10, 20, 30, 40, and 60. (A) Polyarginine 5/pDNA/Ca$^{2+}$ complexes, (B) polyarginine 7/pDNA/Ca$^{2+}$ complexes, (C) polyarginine 9/pDNA/Ca$^{2+}$ complexes, and (D) polyarginine 11/pDNA/Ca$^{2+}$ complexes. (M) Refers to marker and (C) refers to control (pDNA).
Figure 3.
Particle size (effective diameter) of polyarginine 5, 7, 9, and 11/pDNA complexes with different calcium chloride concentrations (0, 150, 300, and 600 mM) at (A) N/P ratio 5, and (B) N/P ratio 10. Results are presented as mean ± SD (n= 3).
Figure 4.
The effect of different calcium chloride concentrations on the zeta potential of PEI/pDNA complexes and polyarginine 5, 7, 9 and 11/pDNA complexes. Results are presented as mean ± SD (n = 3).
Figure 5.
The transfection efficiency was determined for polyarginine/pDNA complexes at N/P ratios of 5, 10, 20, and 30 with different concentrations of added CaCl$_2$ (50, 100, 150, 300, and 600 mM). (A) Polyarginine 5/pDNA/Ca$^{2+}$ complexes, (B) polyarginine 7/pDNA/Ca$^{2+}$ complexes, (C) polyarginine 9/pDNA/Ca$^{2+}$ complexes, (D) polyarginine 11/pDNA/Ca$^{2+}$ complexes. PEI (N/P ratio 10) was used as a standard. RLU$s$ refers to relative light units. Results are presented as mean ± SD (n = 4). The $p$ of different polyarginine complexes were compared with PEI complexes (* = $p < 0.0001$, + = $p < 0.008$, and x = $p < 0.09$, t test).
Figure 6.
The transfection efficiency was determined for polyarginine/pDNA complexes at N/P ratios of 5, 10, 20, and 30 with different concentrations of added CaCl$_2$ (100 and 150 mM) for (A) polyarginine 5/pDNA/Ca$^{2+}$ complexes and (B) polyarginine 7/pDNA/Ca$^{2+}$ complexes. PEI (N/P ratio 10) was used as a standard. RLUs refers to relative light units. Results are presented as mean ± SD (n = 4), (* = p < 0.0001, + = p < 0.005, and x = p < 0.05, one-way ANOVA, Tukey post test).
Figure 7.
The transfection efficiency was determined for pDNA/Ca^{2+} complexes and compared to polyarginine 7/pDNA/Ca^{2+} complexes with different concentrations of added CaCl_{2} (50, 100, 150, 300, and 600 mM), (+ = p < 0.005, and x = p < 0.05, one-way ANOVA, Tukey post test).
Figure 8.
Polyarginine peptide 5, 7, 9 and 11 showed negligible cytotoxicity in comparison to PEI. Viability is expressed as a function of peptide concentration. Results are presented as mean ± SD (n = 3).
Figure 9.
Representative images of A549 cells after incubation of PEI/pDNA complexes and polyarginine 7/pDNA/Ca$^{2+}$ complexes. (A) PEI/pDNA complexes without calcium after 5 hr. (B) PEI/pDNA complexes without calcium after 48 hr. (C) Polyarginine 7/pDNA complexes with 50 mM calcium chloride after 5 hr. (D) Polyarginine 7/pDNA complexes with 50 mM calcium chloride after 48 hr.
Figure 10.  
The mass of protein after 48 hr in cell cultures treated with polyarginine/pDNA complexes at N/P ratios of 5, 10, 20, and 30 with different concentrations of added CaCl$_2$ (50, 100, 150, 300, and 600 mM). The protein level of different polyarginine/pDNA complexes was typically higher than PEI/pDNA complexes.  
(A) Polyarginine 5/pDNA/Ca$^{2+}$ complexes, (B) polyarginine 7/pDNA/Ca$^{2+}$ complexes, (C) polyarginine 9/pDNA/Ca$^{2+}$ complexes, (D) polyarginine 11/pDNA/Ca$^{2+}$ complexes.
Table 1

Sequence and size of polyarginine 5, 7, 9 and 11.

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