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Calcium condensation of DNA complexed with cell-penetrating peptides offers efficient, noncytotoxic gene delivery

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Abstract

Drug delivery strategies using cell penetrating peptides (CPPs) have been widely explored to improve the intracellular delivery of a large number of cargo molecules. Electrostatic complexation of pDNA using CPPs has been less explored due to the relatively large complexes formed and the low levels of gene expression achieved when using these low molecular weight polycations as DNA condensing agents. Here, condensing nascent CPP polyplexes using $CaCl_2$ produced small and stable nanoparticles leading to gene expression levels higher than observed for control PEI gene vectors. This simple formulation approach showed negligible cytotoxicity in A549 lung epithelial cells and maintained particle size and transfection efficiency even in the presence of serum.

Keywords

Gene Delivery; Plasmid DNA; Cell-penetrating peptides; A549 cells

Introduction

Nucleic acid therapeutics continue to offer promise for the treatment of both acquired and inherited diseases. One major obstacle impeding the successful application of nucleic acid drugs is the difficulty to develop a simple, safe, and efficacious delivery system [1–3]. Gene delivery vectors must compact genetic material into nanoparticles that are colloidally stable, protect nucleic acids from enzymatic degradation, effectively transit nucleic acids to target cells, and achieve a significant transfection yield. Viral vectors remain the most effective method of gene delivery even though problems such as immunogenicity remain a concern.

Polymer-mediated gene delivery has emerged as a viable alternative to viral transfection due to potential attributes such as low immunogenicity, low toxicity, ease of synthesis and low cost [4–6]. Many studies have shown that DNA complexes electrostatically with polycations to form "polyplexes" that are endocytosed by many cell types and deliver DNA with varying degrees of efficiency and toxicity [7–16]. Frequently, the most effective polyplexes are also the most toxic, thus hampering clinical translation [1–6]. As a primary example, polyethylenimine

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(PEI) exhibits efficient gene delivery but is also very cytotoxic [17,18]. Cell-penetrating peptides (CPPs) offer a potential alternative to PEI. These short polycations achieve intracellular access by crossing the plasma membrane directly or by endocytosis [19–25] while typically exhibiting low cytotoxicity [26,27]. Covalently conjugating CPPs to gene vectors (e.g. liposomes, polymer nanoparticle, etc.) has shown some promise, but polyplexes of CPPs and DNA have proven to be relatively inefficient and require improvement [28,29].

To provide a simple method for improving the gene delivery of CPP polyplexes, calcium was used to condense large and inefficient CPP polyplexes. The resulting nanoparticles transfected human lung carcinoma cell line A549 more efficiently than PEI and exhibited very low cytotoxicity. Four representative CPPs were studied; Arginine 7 (Arg7), Arginine 9 (Arg9), Antennapedia Heptapeptide (Ahp) and Antennapedia Leader Peptide (Alp) (Table 1). Plasmid DNA encoding firefly luciferase (pGL3, 4.8 kbp) was used as a reporter.

Experimental Section

Materials

Plasmid DNA encoding firefly luciferase (pGL3, 4.8 kbp) was obtained from Promega (Madison, WI). Cell penetrating peptides (CPPs) were purchased from Pi Proteomics (Huntsville, AL). Branched polyethylenimine (PEI, 25 kDa) was obtained from Aldrich (Milwaukee, WI). Calcium chloride ($CaCl_2$. $2H_2O$) was purchased from Fisher Scientific (Pittsburgh, PA). A549 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cell culture medium (Ham's F-12 Nutrient Mixture, Kaighn's modified with L-glutamine) was purchased through Fisher Scientic. Fetal bovine serum (FBS) was purchased from Hyclone. Penicillin-streptomycin was purchased from MB Biomedical, LLC. Trypsin-EDTA was purchased through Gibco. MTS reagent [tetrazolium compound; 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] was purchased from Promega.

Preparation of CPP/pGL3 complexes

CPP/pGL3 complexes were prepared by adding 10 μL (0.1 μg/μL) of pGL3 to 15 μL CPP solution while pipetting. To this solution, 15 μL of known molarity (e.g. 113 mM) $CaCl_2$ was added and mixed by vigorous pipetting. Before performing experiments, the complexes were allowed to equilibrate for 20 min at 4° C.

Preparation of PEI/pGL3 complexes

PEI/pGL3 complexes were prepared by adding 10 μl (0.1 μg/μL) of pGL3 solution to 15 μL (N/P ratio 10) PEI solution while pipetting followed by 20 min incubation at 4° C. Complexes were freshly prepared before each individual experiment.

Characterization of CPPs and PEI complexes

The size and zeta potential of the different complexes were measured by using ZetaPALS dynamic light scattering (DLS) (Brookhaven, Holtsville, NY).

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Cell culture

Culturing of human epithelial lung cell line A549 was performed according to the protocol provided by the American Type Culture Collection. A549 cells were grown in F-12K supplemented with 10% v/v FBS and 1% v/v Penicillin/streptomycin at 37° C in a humidified air atmosphere containing 5% CO_2.

In vitro cell transfection studies

A549 cells were trypsinized, counted and diluted to a concentration of approximately 80,000 cells/ mL. Then 0.1 mL of that dilution was added to each well of a 96-well plate and the cells were incubated in a humidified atmosphere at 5% CO_2 and 37°C for 24 h. Immediately before transfection, the cells were washed once with PBS and 100 μl sample (20% of complex to 80% of serum free cell culture medium) was added to each well. Cells were incubated with the complexes for 5 h. The transfection agent was then removed by aspiration and 100 μL of fresh serum medium was added followed by further incubation. The Luciferase Assay System from Promega was used to determine gene expression following the manufacturer's recommended protocol. The light units were normalized against protein concentration in the cells extracts, which were measured using the BCA™ Protein Assay (Thermo Scientific). The transfection results were expressed as Relative Light Units (RLU) per mg of cellular protein.

Assessment of cytotoxicity (MTS Assay)

Cytotoxicity of polymers was determined by the CellTiter 96® Aqueous Cell Proliferation Assay (Promega). A549 cells were grown as described in the transfection experiments. Cells were treated with the samples for ~24 h. The media were then removed and replaced with a mixture of 100 μL fresh culture media and 20 μL MTS reagent solution. The cells were incubated for 3 h at 37°C in the 5% CO_2 incubator. The absorbance of each well was then measured at 490 nm using a microtiter plate reader (SpectraMax, M25, Molecular Devices Corp., CA) to determine cell viability.

Statistical analysis

Statistical evaluation of comparing the significance of the difference in expression between the means of two groups was performed using the t-test, a value of $p < 0.05$ was accepted as significant.

Results and Discussion

Peptides offer a highly attractive feature of incorporating various biological activities required for biomedical applications[30–34]. Cell-penetrating peptides, a group of short peptides with the potent ability to translocate across the plasma membrane of the cells, have been reported to mediate plasmid DNA delivery into cells[35–37]; however, improving the transfection efficiency of their DNA complexes remains a major challenge. The relatively low transfection level of certain complexes may be due to inadequate escape from endosomes or the inefficient release of DNA from the complexes[38]. Previous studies demonstrated that the HIV-1 TAT peptide could only provide a high level of gene expression when chloroquine (an endosomolytic agent) was added, which is not feasible for

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gene delivery *in vivo*[28,39–41]. One approach for overcoming this limitation was to link the CPPs to produce high molecular weight polypeptides[42–44] or to directly conjugate the CPPs using histidine or cysteine residues[38,45]. Others have tried a low molecular weight PEI with covalently linked TAT to overcome the poor transfection efficiency of the CPP alone[39]. Targeting studies have also been explored. For example, the YIGSR pentapeptide, known to target cell surface laminin receptors, or the LK15 peptide was conjugated to TAT. In each case, the transfection efficiency improved, but gene expression levels were still low compared to PEI[46–48].

In studies reported here, CPP/pGL3 complexes were synthesized by rapidly adding pGL3 to CPP. These complexes were thoroughly mixed by pipetting and then $CaCl_2$ was added to decrease the large size of these complexes. Calcium was previously reported to interact with both amines (e.g. on CPPs) and pDNA within polyplexes to form compact complexes through "soft" crosslinks. Here, the reduction in the size of CPP/pGL3 complexes likely led to some of the noted increase in transfection. A $CaCl_2$ concentration of 113 mM consistently produced small (100–140 nm) and stable CPP polyplexes with a single particle population (polydispersity < 0.23). In general, the zeta potential of CPP polyplexes increased significantly from 8 to 26 mV with increasing concentration of $CaCl_2$ (Figure 1). The CPP polyplexes synthesized with $CaCl_2$ remained stable in the absence and presence of 10% FBS at 37° C for 1 hr. Conversely, CPP polyplexes without added calcium remained large (Figure 2).

A successful gene delivery vector should be able to deliver gene to the cell without negatively affecting the viability of the host cell. To investigate whether the CPPs affected the viability of A549 human lung carcinoma cells, an MTS cytotoxicity assay of free CPP or branched PEI (25 kDa) was conducted. A549 cells were incubated with up to 5 mg/mL of CPPs or PEI for ~24 hrs. Cytotoxicity profiles of Arg7, Arg9 and Ahp peptides revealed no evidence of cytotoxicity and cells maintained high viability (Figure 3), while Alp peptide showed moderate cytotoxicity (IC_{50} ~2144 μg/mL). Branched PEI induced substantial cytotoxicity (IC_{50} ~35 μg/mL).

Luciferase gene expression was measured 24 h after transfection in order to study the ability of CPP polyplexes to transfect A549 cells. Different N/P ratios of the CPP or branched PEI (N/P 10) polyplexes were studied using different concentrations of $CaCl_2$; 0, 28.3, 56.5, and 113 mM as a condensing agent after complex formation. Most CPP polyplexes showed the highest level of gene expression at 113 mM of added $CaCl_2$ for the various N/P ratios when compared to branched PEI, which had excellent transfection efficiency only in the absence of $CaCl_2$ (Figure 4). Arg7, Arg9, Ahp and Alp revealed the greatest transfection efficiency with 113 mM $CaCl_2$ at N/P ratios of 36, 35, 29 and 15 respectively. It is important to note that gene expression was not detectable for CPPs/pDNA complexes without $CaCl_{2.}$

To gain insight into the potential of utilizing calcium condensed CPP polyplexes as delivery vectors in vivo, A549 cells were transfected in the presence of serum (Figure 5). A slight decrease in transfection efficiency was observed and CPP polyplexes exerted a similar reduction in gene expression as that observed for PEI polyplexes. PEI polyplexes have shown effective gene transfection *in vivo*, but is dose limited due to toxicity.

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Conclusion

The delivery of therapeutic nucleic acids by CPP polyplexes condensed with calcium may offer a simple and effective gene delivery method with potential for clinical translation. Adding $CaCl_2$ to CPP polyplexes produced small nanoparticles leading to gene expression levels higher than observed for optimized PEI gene vectors in A549 lung epithelial cells. CPP polyplexes were stable, maintaining particle size in the absence and presence of 10% of FBS over a period of 1 h. The CPPs generally showed negligible cytotoxicity up to 5 mg/mL, which may offer an opportunity to increase the dose of nucleic acid therapeutics to achieve a desired therapeutic effect. The simplicity of the formulation in combination with the efficacy and low cytotoxicity of CPP polyplexes makes them highly interesting vectors for future studies *in vivo.*

Acknowledgments

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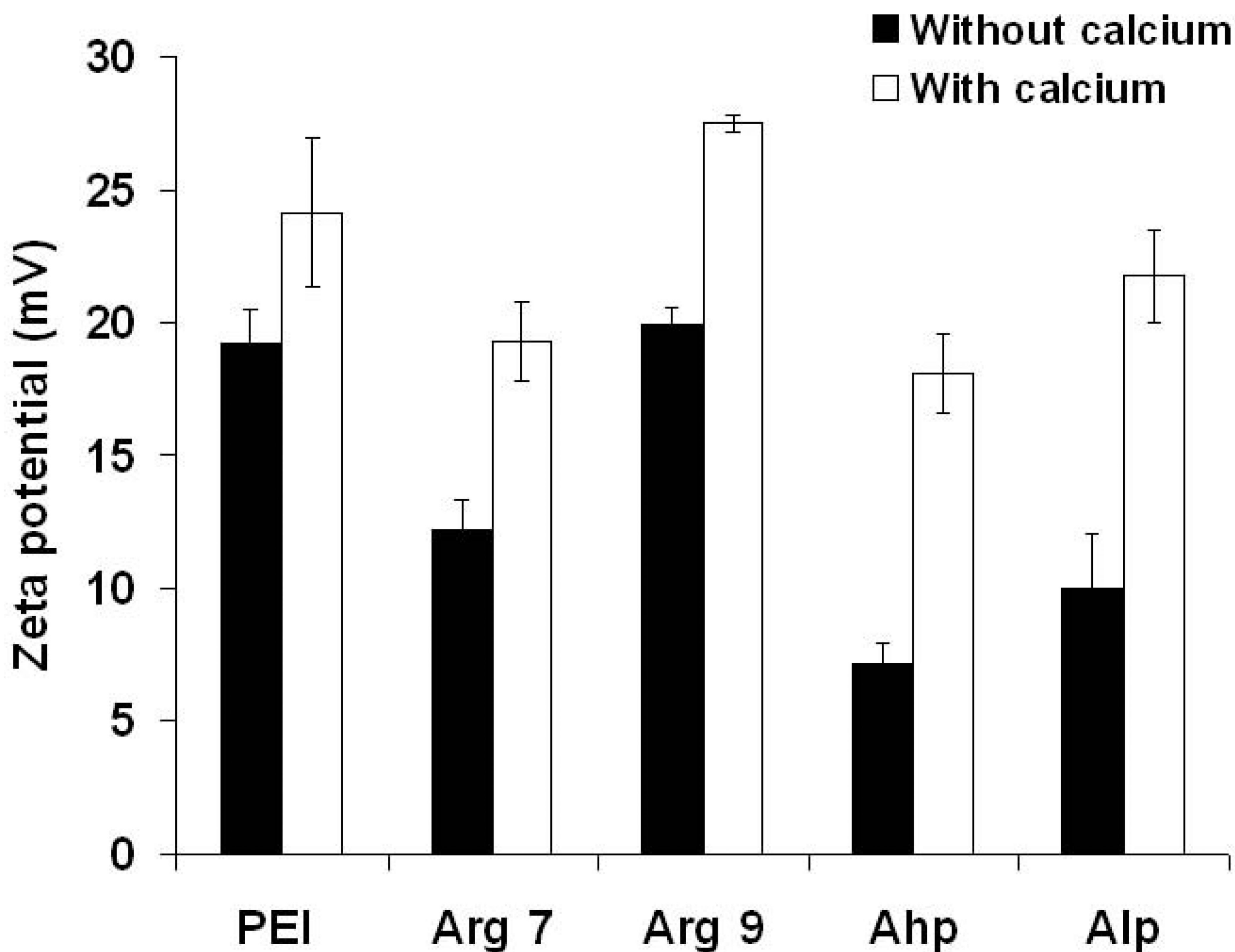

Figure 1.

The effect of CaCl2 (113 mM) on the charge of PEI and CPPs complexes. Results are presented as mean ± SD (n = 3). PEI, polyethylenimine; CPPs, cell-penetrating peptides; Arg7, arginine 7; Arg9, arginine 9; Ahp, antennapedia heptapeptide; Alp, antennapedia leader peptide.

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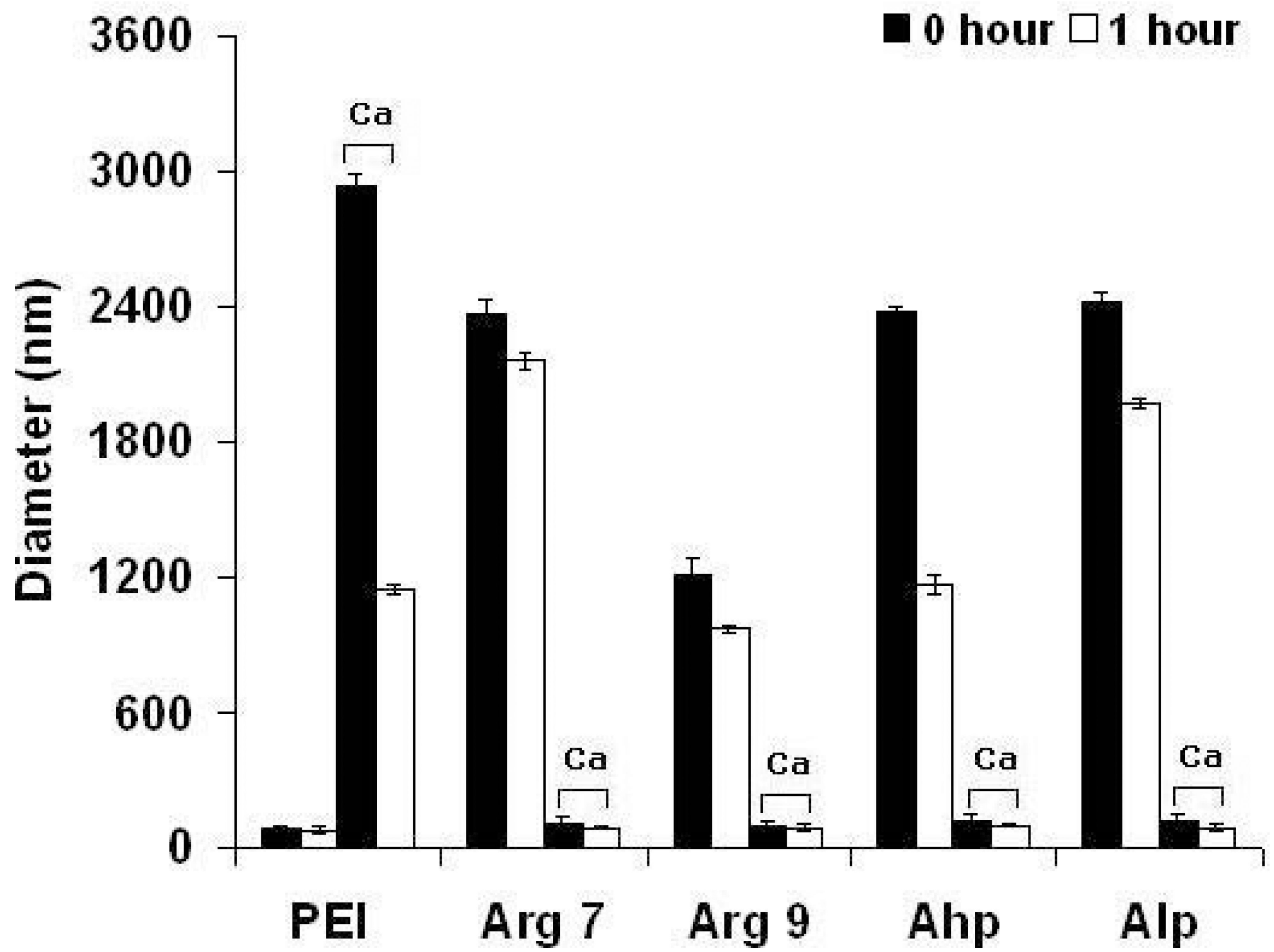

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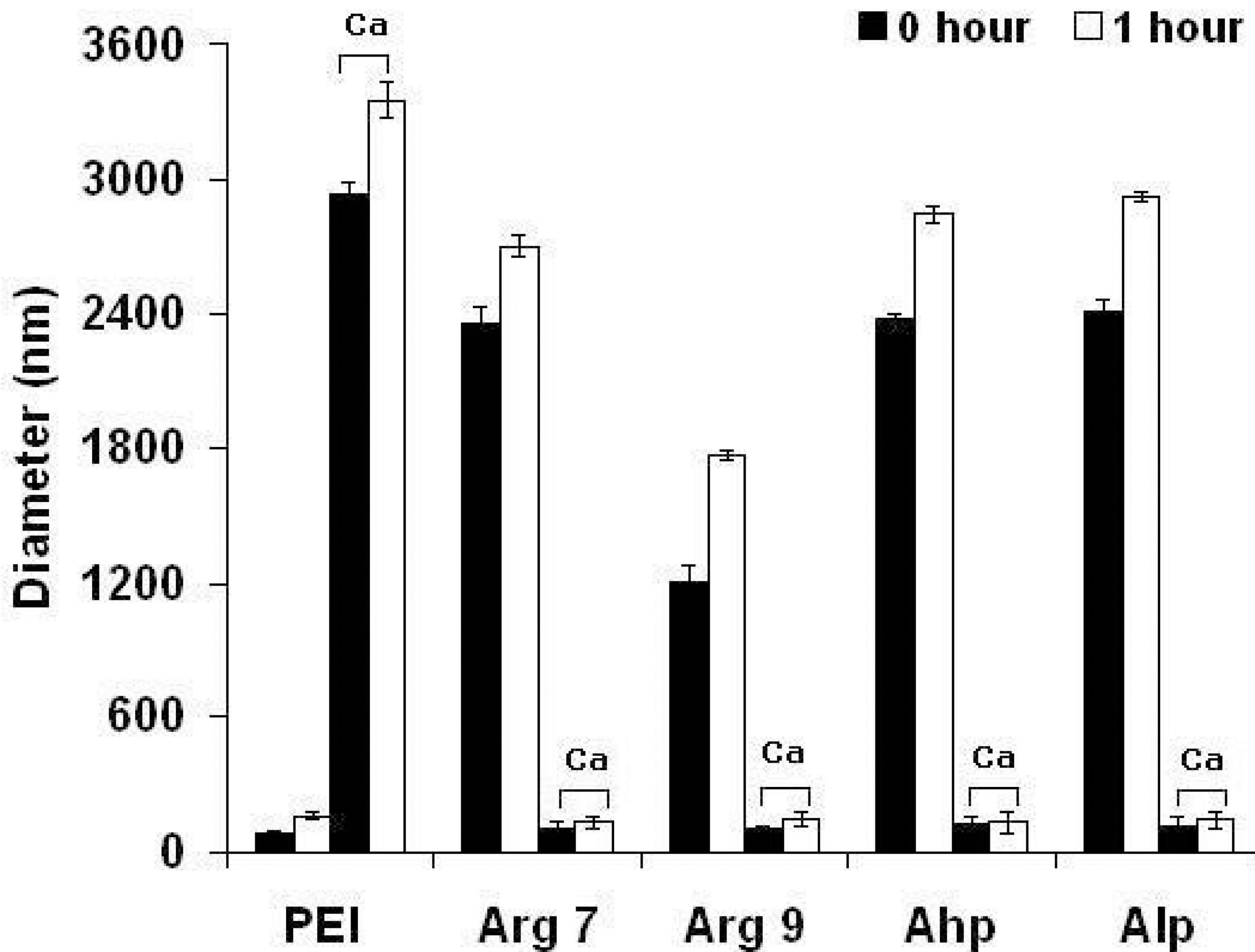

Figure 2.

The diameter of CPPs–Ca/pGL3 and PEI complexes (without and with 113 mM CaCl2) in (a) the presence and (b) absence of 10% fetal bovine serum. Results are presented as mean ± SD (n = 3). PEI, polyethylenimine; CPPs, cell-penetrating peptides; Arg7, arginine 7; Arg9, arginine 9; Ahp, antennapedia heptapeptide; Alp, antennapedia leader peptide.

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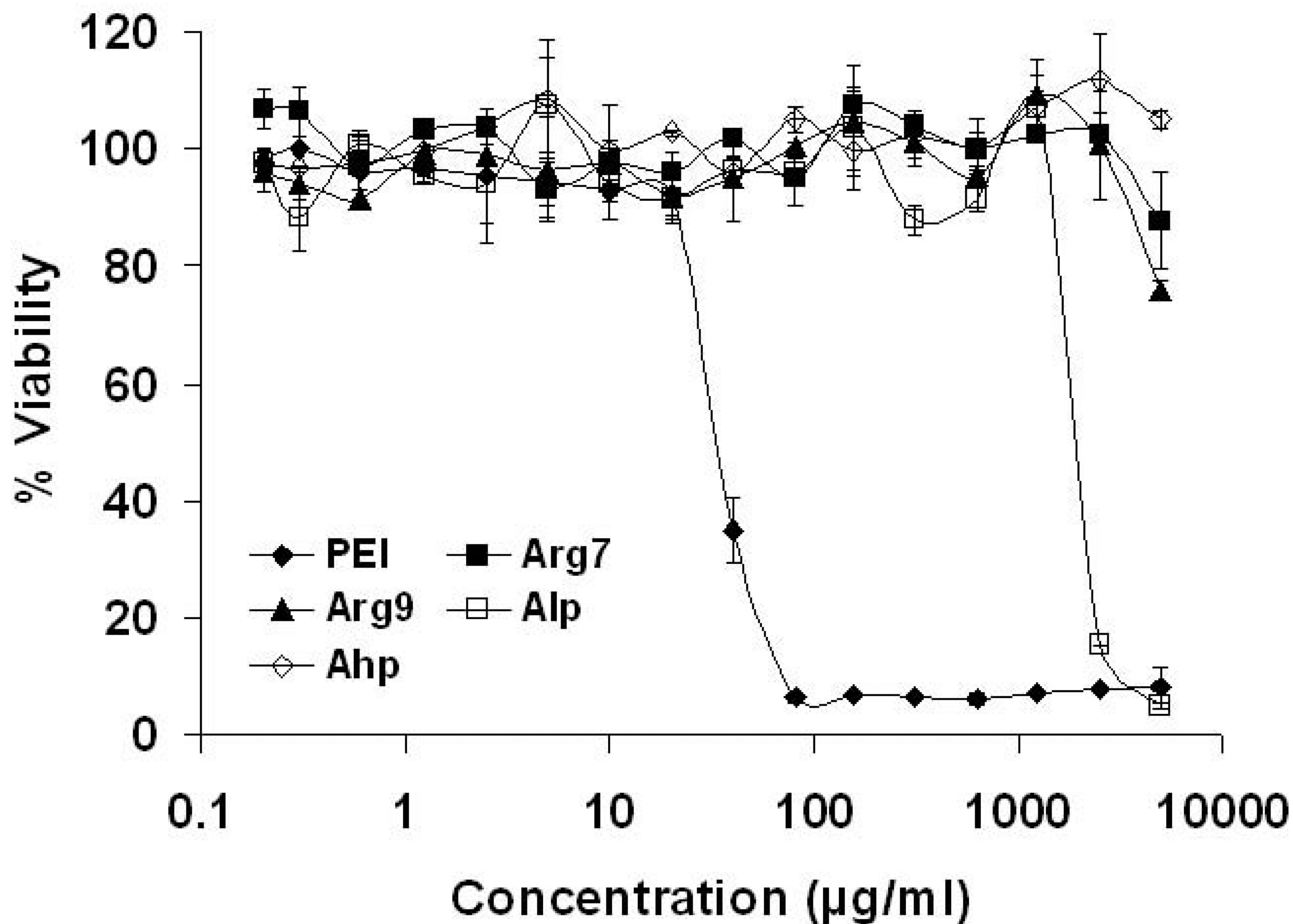

Figure 3.

Cytotoxicity profiles of PEI andCPPs.. Viability is expressed as a function of polymer concentration. Results are presented as mean ± SD (n = 3). PEI, polyethylenimine; CPPs, cell-penetrating peptides; Arg7, arginine 7; Arg9, arginine 9; Ahp, antennapedia heptapeptide; Alp, antennapedia leader peptide.

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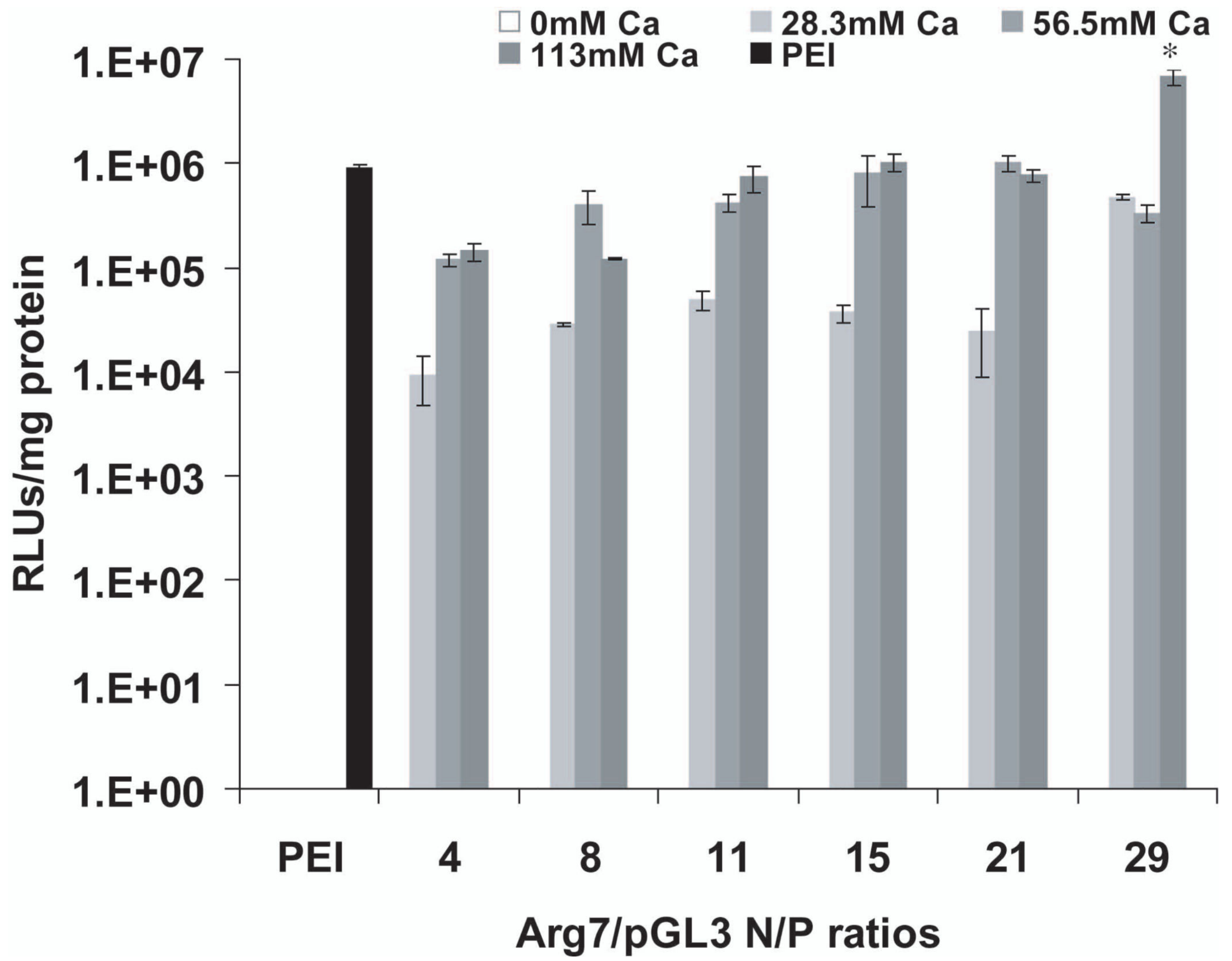

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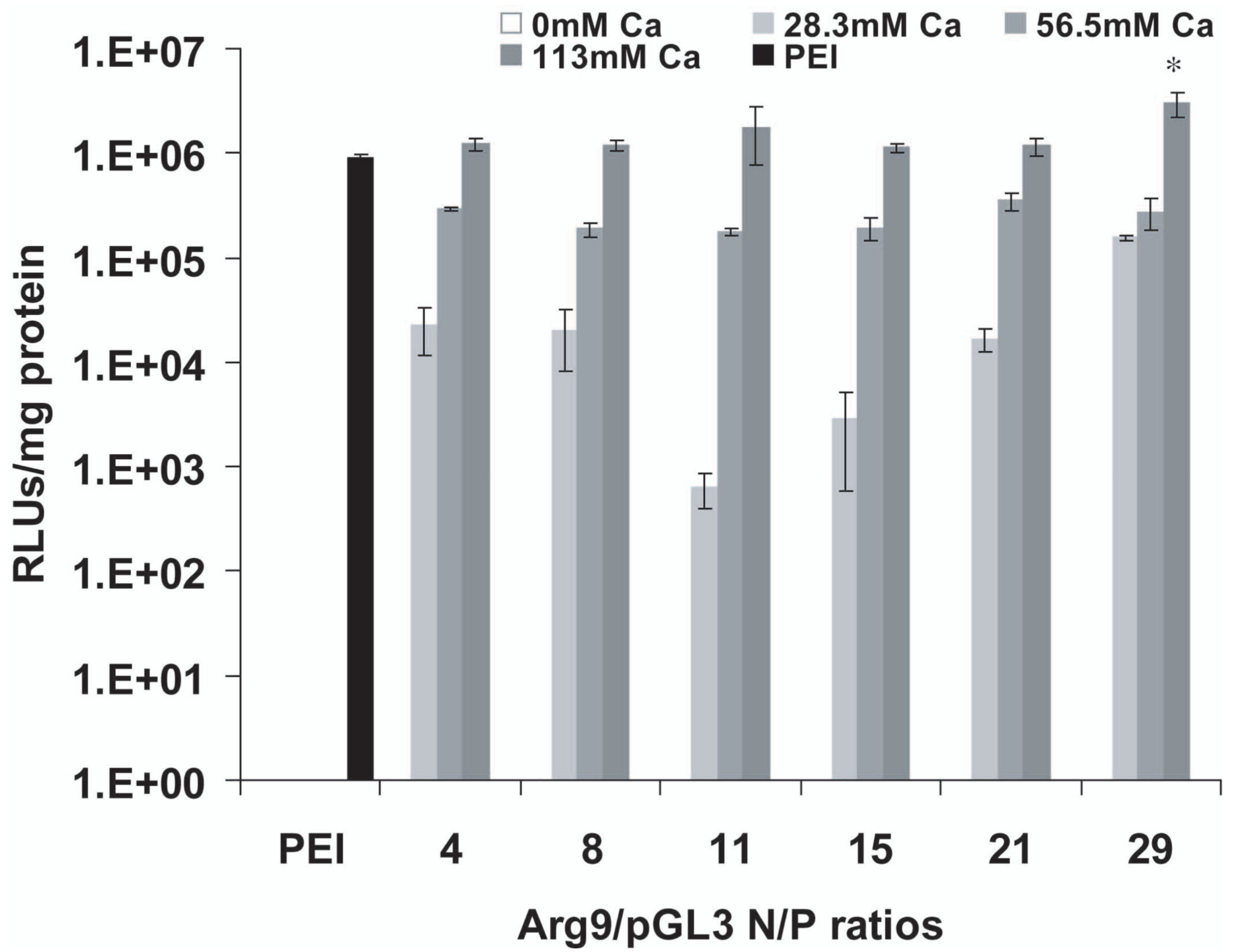

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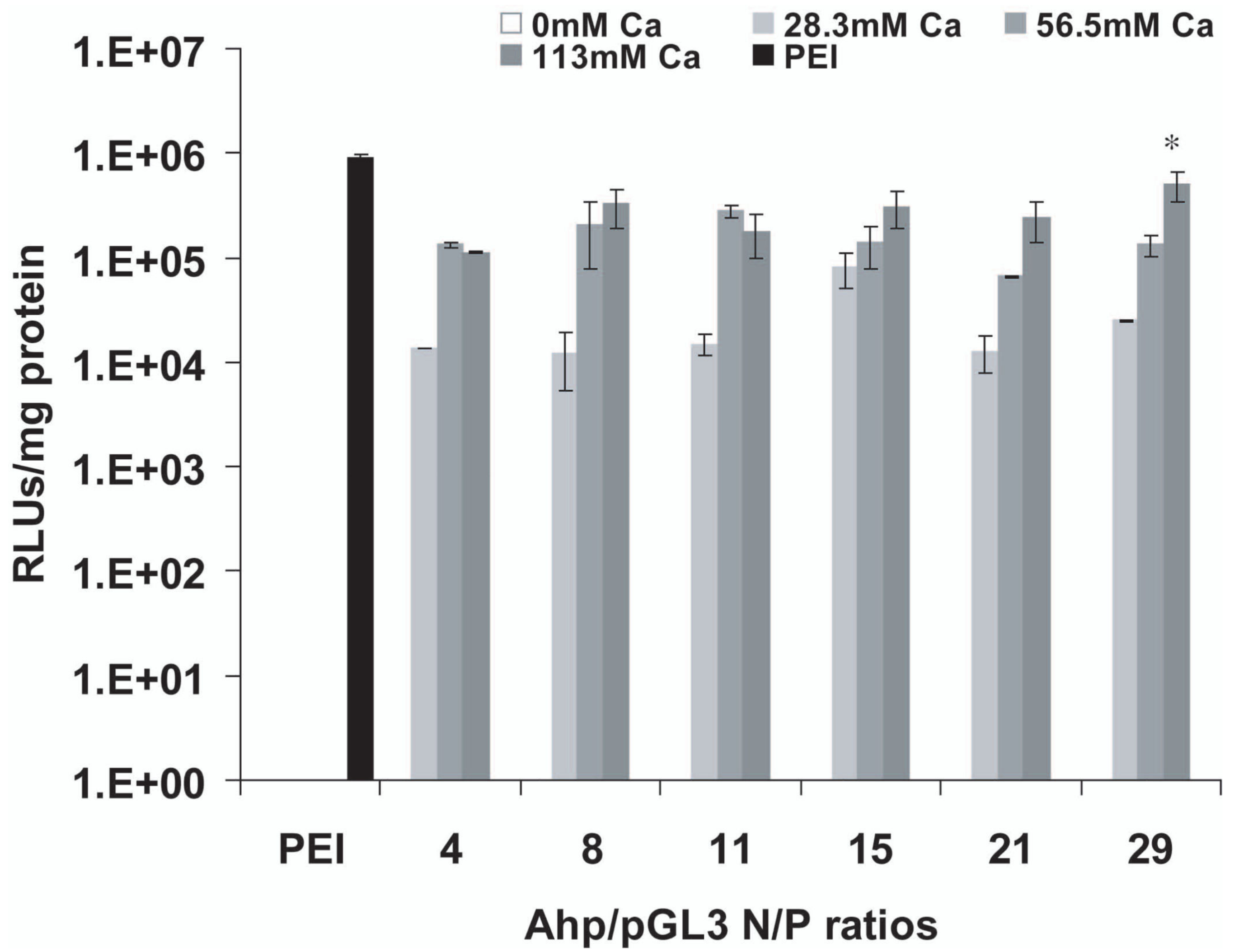

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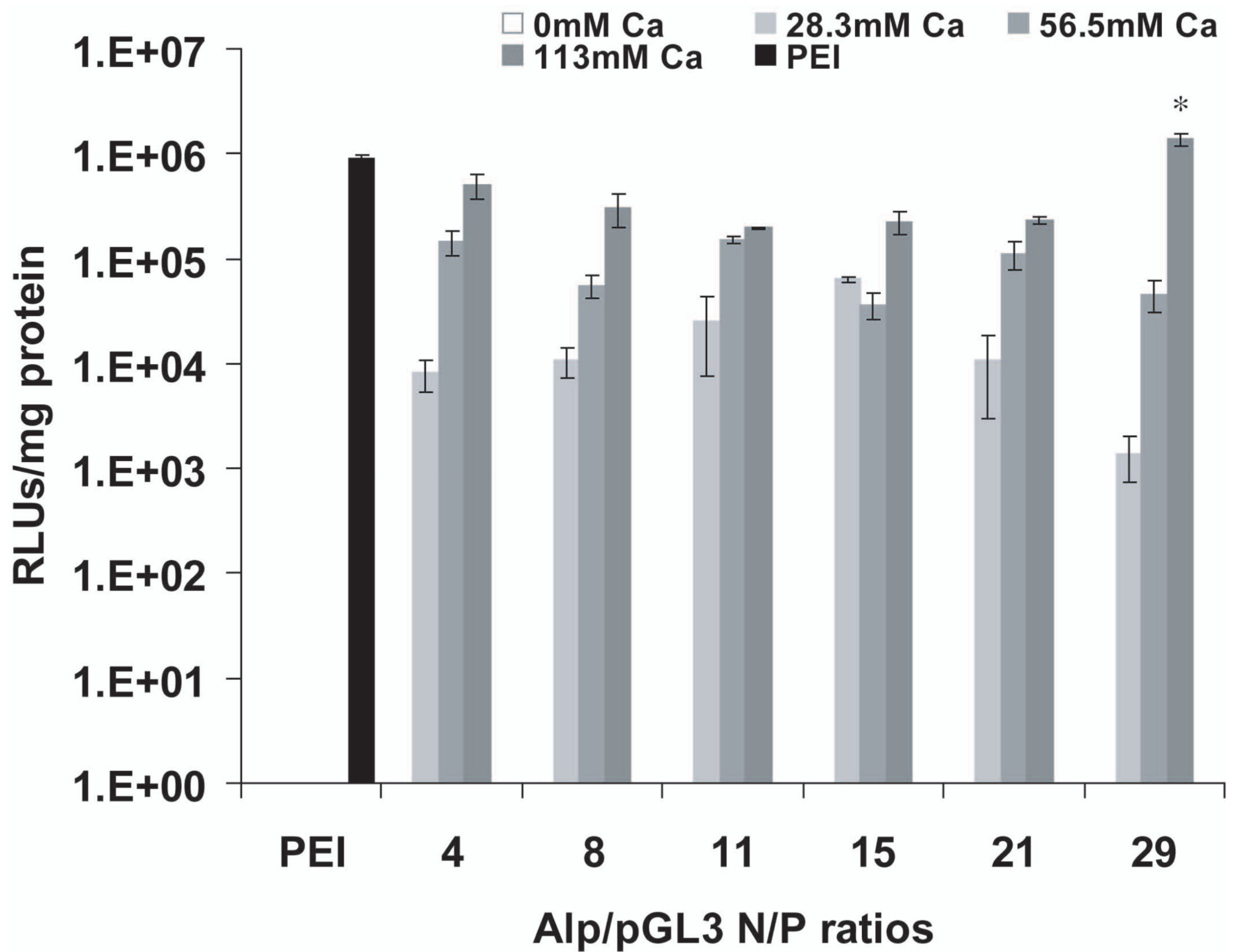

Figure 4.

The transfection efficiency of CPP polyplexes using (a) Arg7, (b) Arg9, (c) Ahp, or (d) Alp with different concentrations of added CaCl2. Results are presented as mean ± SD (n = 3), *$p < 0.001$(Arg7, Ahp, and Alp), and *$p < 0.01$ (Arg9) as compared with PEI. PEI, polyethylenimine; CPPs, cell-penetrating peptides; Arg7, arginine 7; Arg9, arginine 9; Ahp, antennapedia heptapeptide; Alp, antennapedia leader peptide; RLUs, relative light units.

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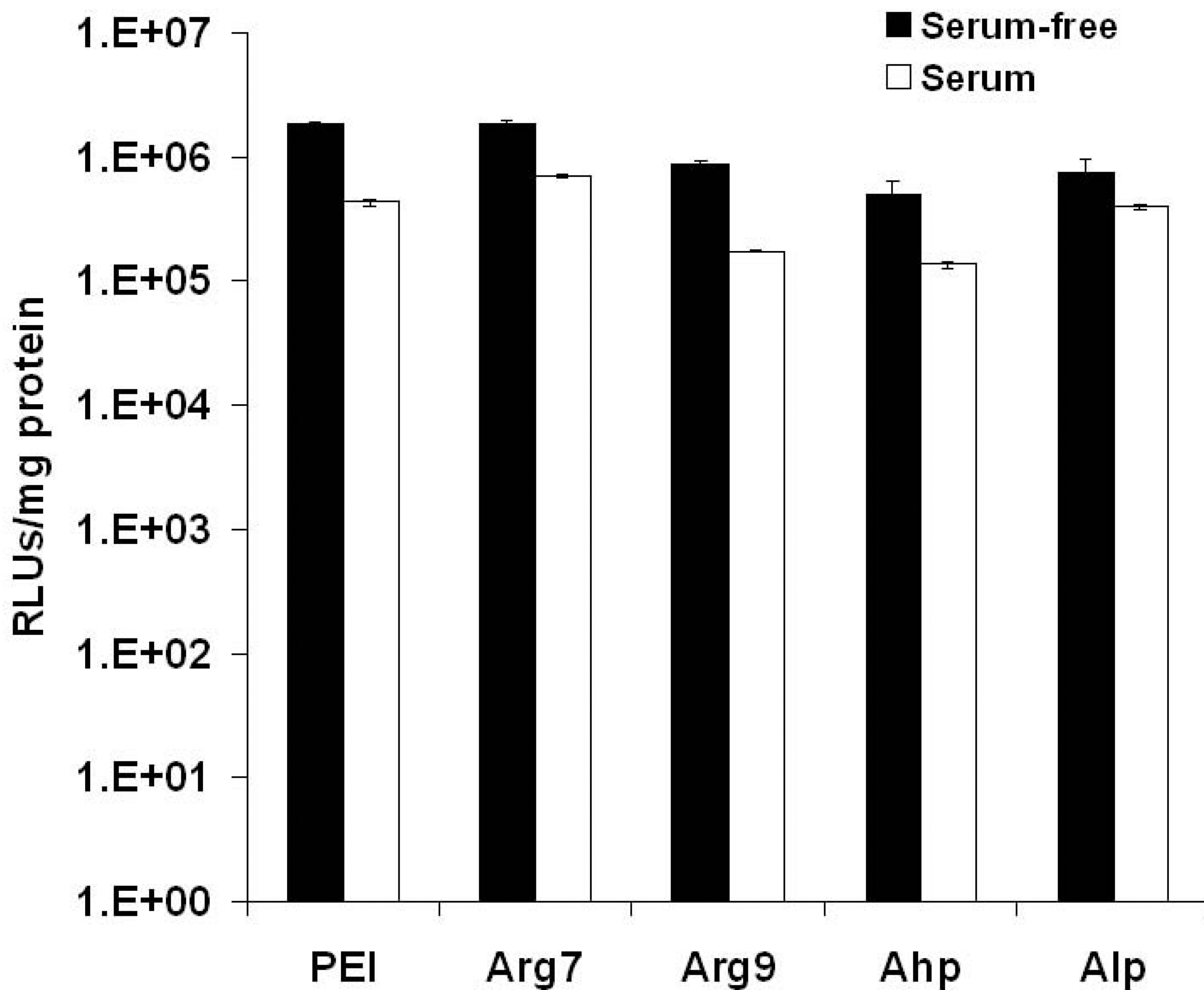

Figure 5.

The transfection efficiency of CPP polyplexes in the absence and presence of 10% fetal bovine serum. Results are presented asmean ± SD (n = 3). PEI, polyethylenimine; CPPs, cell-penetrating peptides; Arg7, arginine 7; Arg9, arginine 9; Ahp, antennapedia heptapeptide; Alp, antennapedia leader peptide; RLUs, relative light units.

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Table 1

Structure of CPPs, Arginine 7 (Arg7), Arginine 9 (Arg9), Antennapedia Heptapeptide (Ahp) and Antennapedia Leader Peptide (Alp).

CPP	Sequence	Molecular weight (Da)
Arg7	RRRRRRR	1,111.3
Arg9	RRRRRRRRR	1,423.3
Ahp	RRMKWKK	1,032.6
Alp	KKWKMRRNQFWVKVQRG	2,276.2