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ARTICLE TYPE

Efficient gene and siRNA delivery with cationic polyphosphoramide with amino moieties in main chain

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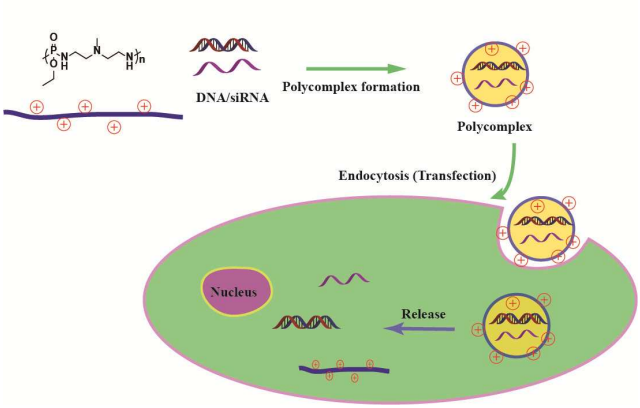
Although a series of cationic polymers were designed and synthesized by various kinds of strategies, the lower transfection efficiency and higher cytotoxicity are still major problem for successful gene therapy. In this study, a novel cation polyphosphoramide (denoted as PPA) with amino moieties in the main chain was synthesized by polycondensation of ethyl dichlorophosphate with N^1-(2-aminoethyl)-N^1-(1-methyl)-1, 2-ethanediamine and investigated as non-viral vectors for gene transfection in target cells. Gel retardation analysis showed that PPA could efficiently retard mobility of DNA at lower N/P ratio. The size and *zeta* potential measurements found that these PPA/pDNA polycomplexes exhibited a decreased size and increased *zeta* potential. The cytotoxicity assay further revealed that PPA was non-toxic to different cells even at higher concentration. It was also observed that polyionic complexes at lower ratio of PPA/DNA (3:1) exhibited higher transfection efficiency. Interestingly, protein phosphorylation and luciferase reporter assay showed that overexpression of target gene (GFP-PKD2) transfected with PPA could enhance phosphorylation of PKD2, downstream I κ B degradation and luciferase reporter activity of NF- κ B signalling pathway in response to PMA , agonist for PKD2 and NF- κ B activation, indicating that the synthesized PPA could effectively deliver more than one exogenous genes into target cells and their proteins showed functional role in target cells. More importantly, Western blotting and immunofluoescence staining of NF-κB p65 nuclear translocation showed that siRNA could be also delivered by PPA effectively, and exhibited silencing the target gene as well as the signaling pathway in A549 cancer cells and HEK293 cells. These results suggested that this novel cation polyphosphoramide could be used as efficient carriers for plasmid and siRNA in future gene therapy applications.

Introduction

Gene therapy has become a promising therapeutic modality for the treatment of genetic and acquired disorders[1]. Non-viral approaches as alternative gene transfer vehicles to the popular viral vectors have received significant attention because of their favourable properties, including lack of immunogenicity, low toxicity, and potential for tissue specificity. Out of these approaches, the most promising and latest systems include cationic polymer-based non-viral gene carriers, dendrimers etc[2, 3] Although therapeutic benefit has been demonstrated in animal models, gene delivery efficiency of the non-viral approaches remains to be a key obstacle for clinical applications.

Cationic polymeric gene carriers mediate gene transfer via condensing and protecting the plasmid DNA in nanoparticle formation, and facilitating cellular internalization and intracellular trafficking of the DNA nanoparticles. To date, a wide variety of polycationic gene carriers have been investigated, including polyethylenimine (PEI)[4, 5], poly(L-lysine)[6, 7], polyamidoamine dendrimers[8, 9], polyaspartamide[10], poly(beta-amino ester[11]), cationic derivatives of polymethacrylate[12]. Recently, phosphorus containing cationic polymers have attracted much attention, due to their potential biodegradability, good biocompatibility and gene/drug carrier properties[13-17]. A series of polyphosphoesters with repeated phosphoester linkage in the backbone have been synthesized by ring-opening polymerization, polycondensation, transesterfication, and enzymatic polymerization[18-20]. Wang and coworker conjugated amine moieties through a phosphate bond in the side chains of polyphosphoesters to prepare polyphosphoesters containing amine groups which demonstrated lower cytotoxicity compared with polyethylenimine (PEI) and poly(L-lysine) (PLL), and showed charge group-dependent transfection abilities and DNA binding capacities[21-23]. Mao and coworker also prepared polyphosphoesters with primary amino group side chains through a phosphoramide bond, found obtained polyphosphoesters exhibit higher transfection efficiencies than those that carry secondary, tertiary or quaternary amino group side chains[23-26].

In this article, we developed a new kind of cation polyphosphoramide (denoted as PPA) with amino moities in the main chain by polycondensation of ethyl dichlorophosphate with N^1-(2-aminoethyl)-N^1-(1-methyl)-1,2-ethanediamine, and then evaluated the gene and siRNA delivery efficiency (scheme 1). It is anticipated that PPA could form polyionic complexes with DNA/siRNA *via* electrostatic interaction. After characterizing the chemical structures of polymer, as well as the properties of polyionic complexes, we further explored the capability of PPA as the delivery carriers of DNA. Our results showed that PPA could effectively deliver one and more exogenous genes into target cells and transfected genes by PPA also showed physiological role in the target cells. More importantly, Western blotting and immunofluoescence staining of NF-κB p65 nuclear translocation showed that siRNA could be also delivered by PPA effectively, and exhibited silencing the target gene as well as the signaling pathway in A549 cancer cells and HEK293 cells.



Scheme 1 Schematic illustration of the polyplex formation and transfection in cell.

Materials and Methods

Materials

Ethyl dichlorophosphate and N^1-(2-aminoethyl)-N^1-(1-methyl)-1,2-ethanediamine, THF and Triethylamine used for polymerization and all ultrapure reagents were from Sigma. Lipofectamine 2000 was from Invitrogen, while Hilymax transfection reagent was from Dojindo (JAPAN); Cell counting kit-8 was purchased from Dojindo; PMA (phorbol 12-myristate 13-acetate, TPA, 12-O-tetradecanoylphorbol-13-acetate), DAPI andTNF-α from Sigma. Antiobodies for IκB、pS744/748 from Cell signalling Technology (CST), PKD2 antibody from Merker. Immobilon-P membranes were purchased from Millipore; si-PKD2 labeled cy3(si-PKD2-cy3) from GenePharma (shanghai ,China); Plasmid DNA (pEGFP and pEGFP-PKD2) was kindly provided by Dr. QJ Wang in University of Pittsburgh School of Medicine. 2×NF-κB luciferase reporter was kind gift from Dr. Yong Jiang in Southern Medical University. Internal control plasmid (pGL4.74[hRluc/TK] and Dual-Luciferase Reporter Assay kit from Promega.

Characterization.

^{1}H (400 MHz) and ^{31}P (100 MHz) NMR spectra were recorded on a Bruker Avance/DMX 400MHz NMR spectrometer with DMSO-d_6 and tetramethylsilane as an internal reference. IR spectra were measured using a Shimadzu FTIR-8100 spectrophotometer. The number- and weight-average molecular weights (M_n and M_w, respectively) of the polymer were determined using gel permeation chromatography (GPC) on a Jasco Gulliver system (PU-980, CO-965, RI-930, and UV-1570) equipped with polystyrene gel columns (Shodex columns K804, K805, and J806) and calibrated by polystyrene standards at 40 °C using THF as the eluent.

Synthesis of Polyphosphoramide

A typical experimental procedure for synthesis of polyphosphoramide is given below. Under nitrogen protection, N^1-(2-aminoethyl)-N^1-(1-methyl)- 1,2-ethanediamine (1.17 g, 10 mmol), 20 mL of anhydrous THF, and 10 mL of anhydrous triethylamine were consecutively added into a 100 mL freshly flamed and nitrogen-purged flask. After stirring at 0°C for 30 min, ethyl dichlorophosphate(1.62g, 10 mmol) was drop added. The reaction was carried out at room temperature for 48h and the product was obtained by precipitating concentrated mixture into cooled mixture of diethyl ether and methanol (10:1, v/v), and dried under vacuum at room temperature with a yield of 74.3%.

Polymer: GPC (THF, polystyrene standard): M_w=5174, M_n=3618, PDI =1.43.

^{1}H NMR (DMSO-d_6, ppm): δ 4.51~4.72 (m, 2H), 3.08~2.62(m, 11H), 1.31~1.09 (m, 3H).

^{31}P NMR (DMSO-d_6, ppm): δ 10.02.

IR(cm^{-1}, KBr):3371, 2951, 1610, 1588, 1204, 1022, 907.

Polyionic Complexes Formation

All polycomplexes of plasmid DNA or siRNA and polyphosphoramide were freshly prepared before use. The prepared polymer was dissolved in DMSO (stock solution:100μg/μL), and diluted with sterile PBS buffer into 0.5μg/μL and 1.0μg/μL, then mixed with different concentration of plasmid DNA(pEGFP or pEGFP-PKD2) or siRNA, and kept still for 15min to allow the polymer encapsulate plasmid DNA or siRNA.

Particle Size and *zeta* Potential Measurement

The particle size of polyionic complexes were measured by Nano-ZS ZEN3600 (Malvern Instruments, Germany). The *zeta* potential of polyionic complexes were measured using Nano-ZS ZEN3600 based on the principle of phase analysis light scattering. The particles were prepared as described above and the size measurement was performed at 25 °C at a 90° scattering angle, recorded for 180 s for each measurement. The mean hydrodynamic diameter was determined by cumulative analysis. The *zeta* potential measurements were performed using an aqueous dip cell in the automatic mode. All measurements are given as mean values of at least three independent runs and performed in triplicate.

DNA Gel Retardation Assay

The plasmid DNA binding capacity of PPA at various N/P ratios was measured by agarose-gel retardation assay. The mixtures were prepared as described, after 15 min incubation at RT, the loading buffer was added to each mixture and finally was applied to a 1.0% agarose gel containing ethidium bromide, the plasmid DNA bands were visualized using a Tanon 1600 Gel Image System.

Cell Culture

In this study, A549 cells (human lung cancer cell lines), DU145 cells(human prostate cancer cells), HeLa(human cervical cancer cells) ,HEK293 cells (human embryonic kidney cells) were cultured with DMEM medium supplemented with 10% FCS (fetal calf serum), and the cells were maintained in incubator at 37℃ under humidified atmosphere of 5% CO_2.

Cytotoxicity Assay

A Cell Counting Kit (CCK-8) assay was used to evaluate the effect of PPA on the cell cytotoxicity and cell viability. Briefly, prior to cytotoxicity measure, the trypsinization of cells in the logarithmic growth phase were harvested and seeded on a 96-well plate at a density of 0.4 x 10^5/mL, and allowed to adhere overnight, followed by the treatment of 15μg/mL, 30μg/mL of PPA . After 24 h cultivation, the medium were replaced with the same volume of fresh medium containing with 10μL of WST-8 solution (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-

disulfophenyl)-2H-tetrazolium, monosodium salt), and plate was incubated for additional 4h at 37℃. The absorbance of each well at 450 nm was measured using a Bio-Rad model 680 microplate reader. The relative cell viability was calculated as follows:

Cell viability（%）=($OD_{samples}$-OD_{DMSO})/(ODcontrol-OD_{DMSO}x100 . Each concentration was repeated five times respectively.

Plasmid DNA and siRNA Transfection

For *in vitro* transfection study, cells were seeded into a 24-well plate at a density of 3.0×10^5 cells/mL in 500 μL DMEM medium supplemented with 10% FCS, 24 h later, the cells were continuously incubated for 6 h with the plasmid DNA or siRNA complexes mixed in different N/P ratios, then the medium was replaced with fresh medium and continued to incubate for another 24 h. The expressed fluorescent green proteins and efficiency were visualized and recorded on Nikon Ti-s invert fluorescence microscopy. For siRNA transfection, the siRNA sequence of PKD2 labeled cy3 as follows: 5′-CCUGAGUGUGGCUUCUACGGCCUUU-3′ (Invitrogen validated siRNA, Cat. 45-3192)

Western Blot Analysis

In a similar way, transfection was done using plasmid 1.0 μg with polymer in HEK293 cells seeded in 24-well plate at a density of 3.0×10^5 cells/mL, overexpression of PKD2 was measured by Western blot as our previously described[27]. After 24h transfection, medium were replaced with serum-free medium for 16 h, and then treated with PMA (100nM) for 30 min, and the transfected cells were washed with cold PBS twice, total protein was extracted from cells with 2×loading buffer. 15μL cell lysates were separated on 10% Bis-Tris-polyacrylamide gels and then transferred to Immobilon-P membranes (Milipore, Bedford, USA) at 100V for 60min, after incubation in 5% milk containing 2% BSA in PBST for 1h, membranes were incubated in 5%BSA in PBST with antibodies against PKD2(1:2000) overnight, then washed with PBST for 15min/3 times, the membranes was further incubated in 5% milk in PBST with secondary antibodies, respectively, for 1h and visualized using ECL system(Pierce Rockford, USA). Expression of levels of protein was normalized against β-actin protein expression levels. The films were scanned with UMAX powerlook 2010xl and images were processed with smartdraw 2010 software.

Dual-Luciferase Reporter Assay

HEK293 cells were transfected with the plasmids including pEGFP or pEGFP-PKD2 with 2 xNF-κB promoter reporter plasmid and internal control plasmid (pGL4.74[hRluc/TK]), 24 h after transfection the cells were serum starved for 12h and stimulated with PMA (100 nM) for another 16 h. The cells were washed by PBS and lysed with 1xPLB(passive lysis buffer), the relative light units(RLU) of the stimulated cells were measured using the Dual-Luciferase Reporter Assay kit.

Flow cytometry analysis

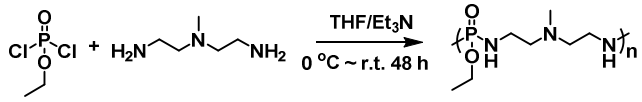
Analysis of GFP fluorescence and cy3-labeled scrambled siRNA content was done by on FACSCalibur (BD, USA). Cells were trypsinized and collected, washed with phosphate-buffered saline (PBS), and then resuspended in 0.2 mL of PBS.transfection were used as a control for background calibration. Results was analyzed by flowjo software.

Indirect Immunofluorescence

Cells were seeded on cover slips in 6-well plate and allowed to attach overnight. Immunofluorescent staining was performed as previously described[28].

Statistical analysis

Statistical analysis of data was performed with GraphPad Prism 5 software. The results were expressed as mean ± SE, and a value of P less than 0.05 was considered statistically significant.

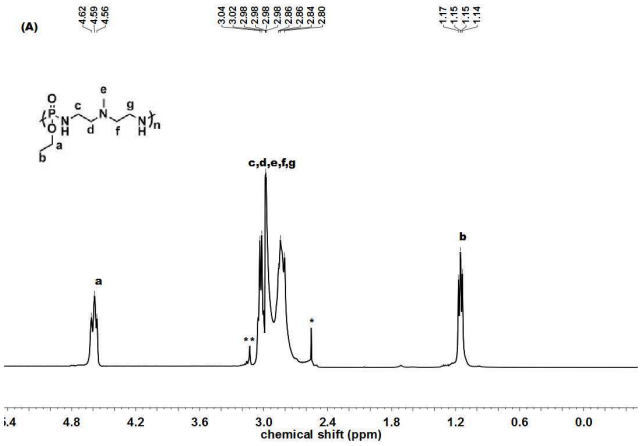


Scheme2 Synthesis of polyphosphoramides

Result and Discussion

Synthesis and characteristic of PPA

Polyphosphoramide were synthesized by N^1-(2-aminoethyl)-N^1-(1-methyl)-1,2-ethanediamine reacted with ethyl dichlorophosphate under N_2 protection(Scheme 2). The successful synthesis of polyphosphoramide was demonstrated by NMR spectra (Figure 1). Resonance at 4.51~4.72 ppm (a) is assigned to methylene protons of PPA (-POCH_2CH$_3$, 2H), while resonance at 1.09~1.31 ppm (b) is assigned to methyl protons (-POCH$_2$CH_3, 3H) of polyphosphoramide. Resonances at 2.62~3.08 ppm (*c,d,e,f,g*) are the characteristic signals of methylene or methyl protons (-NC$\boldsymbol{H}_2$CH_2)N(CH_3) C$\boldsymbol{H}_2$CH_2N-, 11) of the polyphosphoramide main chain. We also analyzed their structures by ^{31}P NMR spectroscopy. The ^{31}P NMR spectrum of polyphosphoramide (Figure 1B) gave a strong resonance at δ 10.02 ppm, assigned to the phosphorus atom in polyphosphoramide block.



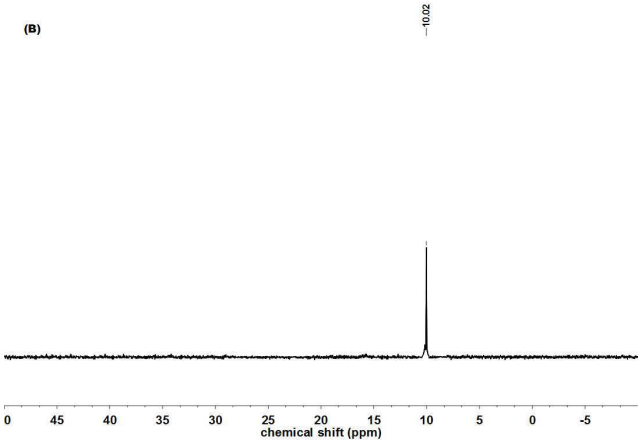


Figure 1 (A) ^{1}H NMR spectra (in DMSO-d_6) of PPA, the solvent and water peaks are marked with asterisks, and (B) ^{31} P NMR spectra (in DMSO-d_6) of PPA .

DNA binding ability

To evaluate the DNA binding ability of PPA, gel retardation assays were performed by electrophoresis of PPA/pDNA polyionic complexes using a 1.0% agarose gel containing ethidium bromide. As shown in Figure 2 A-B, both lower content of PPA (0.5μg/μL) and higher content of PPA (1.0μg/μL) in the same content of pDNA (0.5μg) can complex pDNA well at certain N/P values, the PPA/pDNA was completely retained in the samples at N/P 8 and higher ratio, indicating effective DNA condensation. In comparison, with higher content of pDNA (1.0 μg) and an increase the N/P ratio, the complexes completely lost mobility when formed at a lower N/P value of 4 (Figure.2C), indicating a high complex capacity of full DNA chain with PPA.

Figure 2 Agarose gel electrophoresis retardation assay for PPA/pDNA at different ratio.

Particle size and *zeta* potential measurement for the polyionic complexes

The particle size and *zeta* potential of PPA/pDNA polycomplexes have been shown to influence the cellular uptake, endocytosis pathway, nuclear entry, and transfection efficiency[29, 30]. PPA/pDNA polyionic complexes were prepared by simple and direct mixing of pDNA and PPA solutions at various weight ratios (N/P ratio) in 10 mM Tris–HCl buffer, pH 7.4. The particle size and *zeta* potential of polyionic complexes were measured by Nano-ZS ZEN3600, which were shown in Figure 3 and 4. All the sizes of complexes were measured at weight ratios ranging from 1:1 to 16:1. All complexes were very small with sizes ranging from 100 to 166 nm. The particle sizes decreased slightly with increasing N/P ratio.

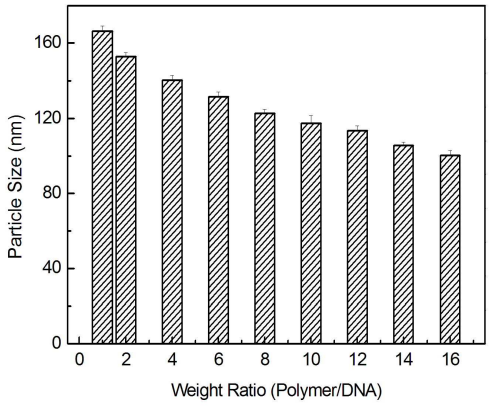


Figure 3 Particle sizes of the PPA/pDNA polycomplexes prepared at different mixing ratios of PPA to pDNA.

Zeta potentials of the polyionic complexes, characterized by a zeta potential analyser with dynamic light-scattering capability, increased from 26.6mV to 38.4mV with the increasing NP ratio (Figure 4). The positive zeta potential of PICs made complexes easier to be uptaken by cells, attributed to the electrostatic interactions between negative characteristic.

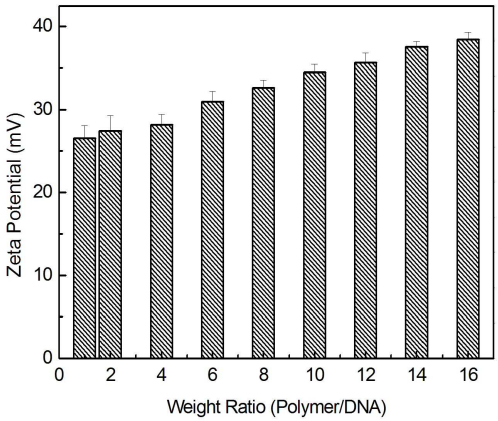


Figure 4 Zeta-potentials of the PPA/pDNA polycomplexes prepared at different mixing ratios of PPA to pDNA

Cytotoxicity of PPA

We further evaluated the cytotoxicity of PPA at different concentration by CCK-8 assay in human lung adenocarcinoma A549 cells , human prostate cancer DU145 cells and HEK293 cells. As shown in Figure 5A, over the tested range of PPA concentration (10-1000μg/ μ L), no significant difference of cell viabilities were observed from A549, DU145 and HEK293 cells compared with DMSO control group, indicating a lower cell cytotoxicity of PPA towards to the different cells even in higher concentration.

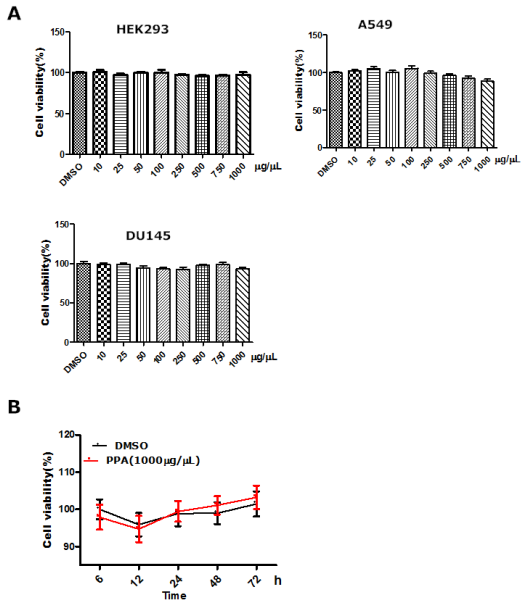


Figure 5 The cytoxicity of PPA at various concentration in different cell lines by CCK-8 assay.(A) Cell viability for PPA in A549 ,DU145 and HEK293 cells. (B) Cell viability for PPA in HEK293 cells were measured for indicated time compared to DMSO control. Percent viability of cells is expressed relative to control cells (n= 5, error bar represents SD).

In order to investigate whether the cell cytotoxicity of PPA was in time dependent manner, HEK293 cells were incubated with higher concentration PPA (1000μg/ μ L) for 6h,12h, 24h,48h,72h, , there was also no significant cell cytoxicity of PPA at 1000μg/ μ L compared to DMSO control at indicated time (Figure 5B). These results suggested that our novel synthesized PPA could be used *in vitro* or *in vivo* as gene carrier with lower cytotoxicity.

Transfection efficiency of plasmids by PPA

We then investigated the *in vitro* transfection efficiency of PPA/plasmid polycomplexes in HEK293cells lines using a pEGFP plasmid, encoding for enhanced GFP protein.The transfection efficiency was visualized by observation of GFP-positive cells using inverted fluorescence microscope, the results showed that the transfection efficiency of PPA at N/P ratio(3:1) were comparable to the commercially available transfection reagents lipofectamine 2000 and Hilymax, showing similar fluorescence intensity for GFP protein in target cells (Figure 6A).Moreover, Flow cytometry was further performed to precisely quantify GFP-positive cells by in HEK293 cells, as shown in Figure 6B, more than 60% (64%)of cell were fluorescence GFP-postitive in PPA transfcetion group , showing comparable transfection to lipofecatmine 2000(62% of GFP-positive cells) and higher transfection than Hilymax(48% of GFP-positive cells), another commercial transfection reagent.

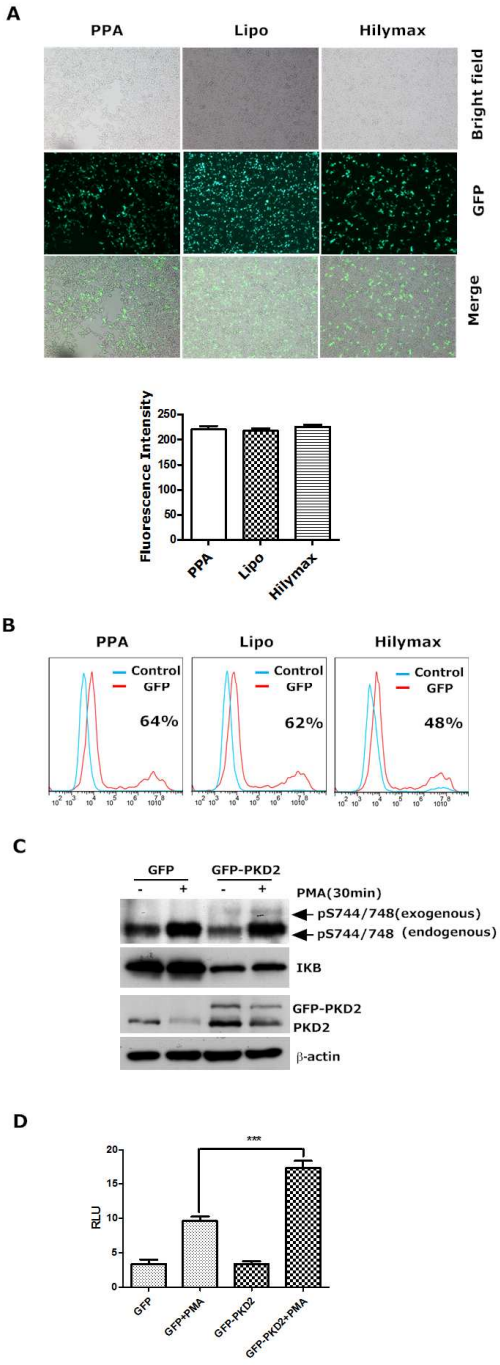


Figure 6 Transfection efficiency of plasmids and phosphorylation activation as well as luciferase activity assay. (A) Comparsion of transfection efficiency of pEGFP plasmid transfected in HEK293 cells using PPA, Lipofectamine2000 and Hilymax, and quantified by Image Pro Plus software analysis; (B) FACS was further performed to assess the transfection efficiency of pEGFP plasmid using PPA, Lipofectamine2000 and Hilymax in HEK293 cells; (C) Overexpression of GFP-PKD2 and its activation assay in response to PMA treatment in A549 cancer cells by Western blot;(D) Luciferase reporter (2x NF-κB) activity assay for HEK293 cells transfected with pEGFP/PPA or GFP-PKD2/PPA polyionic complexes in response to PMA treatment in HEK293 cells, data represent the mean±s.e.m. of three independent experiments(***p<0.001).

Because the phosphorylation of PKD and IκB degradation cascade are the critical event that PKD specific agonist mediated NF-κB signalling passway[31-33], we then explored whether

exogenous PKD2 delivered by PPA can have biological activity and activate downstream target IKB degradation of NF-KB signalling pathway in response to PKD specific agonist phorber ester PMA. As shown in Figure 6C, overexpression of GFP-PKD2 could be found in A549 cancer cells transfected with polyionic complexes containing 1.0μg/μL PPA and 1.0μg GFP-PKD2 at N/P ratio(3:1), indicating that exogenous GFP-PKD2 plasmid could be transfected efficiently and overexpressed in A549 cancer cells, while, overexpression of GFP-PKD2 could enhance the endogenous and exogenous phosphorylation of PKD at active loop(S744/748) and promote IκB degradation of NF-κB signalling passway in response to PMA(100nM) treatment for 30min. These result suggested that our novel synthesized PPA could effectively deliver exogenous gene into target cells and showed functional role of protein expressed by interest of gene in specific signalling passway.

Next, we evaluated whether more than one exogenous genes can also be effectively delivered into target cells by PPA transfection and whether related protein or kinases expressed by these target genes can exhibit biological function in cells, so we cotransfected NF-κB-luc reporter gene, internal control Rellina luciferase plasmid (pGL4.74[hRluc/TK]) with pEGFP or GFP-PKD2 plasmid with PPA (1.0μg/Ml) into HEK293 cells, 24 h after transfection the cells were serum starved for 12h and stimulated with or without PMA (100 nM) for another 16 h. Dual-Luciferase reporter assay showed that PMA-induced DNA-binding activity of NF-κB to a consensus promoter sequence(2X NF-κB) increased remarkably in HEK293 cells transfected with GFP-PKD2 plasmid compared with pEGFP transfected HEK293 cells (Figure 6D), indicating that PKD2 mediated PMA-induced NF-κB transcriptional activation in target cells . Taken together, these results suggested that our synthesized PPA could effectively deliver one and more exogenous genes into target cells and transfected genes by PPA also show physiological role in the target cells.

Transfection efficiency of siRNA by PPA

Synthetic siRNAs are chemically synthesized double-stranded RNAs with structures that mimic the cleavage products of the enzyme Dicer. Upon introduction into the cytoplasm, synthetic siRNAs are incorporated into the RNA interference (RNAi) machinery in the same way as endogenous small RNAs. The great therapeutic potential of siRNA is a result of its ability to silence nearly any targeted gene after introduction into cells[34, 35]. To investigate whether our synthetic novel PPA can deliver the siRNA and silence the target gene of siRNA in cells, siRNA control(siCTL) or siRNA of PKD2 labeled cy3/ (si-PKD2-cy3) were encapsulated with PPA at N/P ratio(3:1) to form a PPA-siRNA complex and then added to Hela cells or HEK293 cells, after 48h, the cell were visualized by fluorescent microscopy and FACS. The result from fluorescent images showed that siRNA PKD2 labeled cy3 transfection by PPA reach a comparable level to that of commercially available transfection reagents lipofectamine 2000 in HEK293 cells and HeLa cells, (Figure 7A). FACS analysis further showed that the transfection efficiency of siRNA by PPA reached up to 56% of HEK293 and 38% of HeLa cells similar to lipofectamine 2000(Figure 7B) .

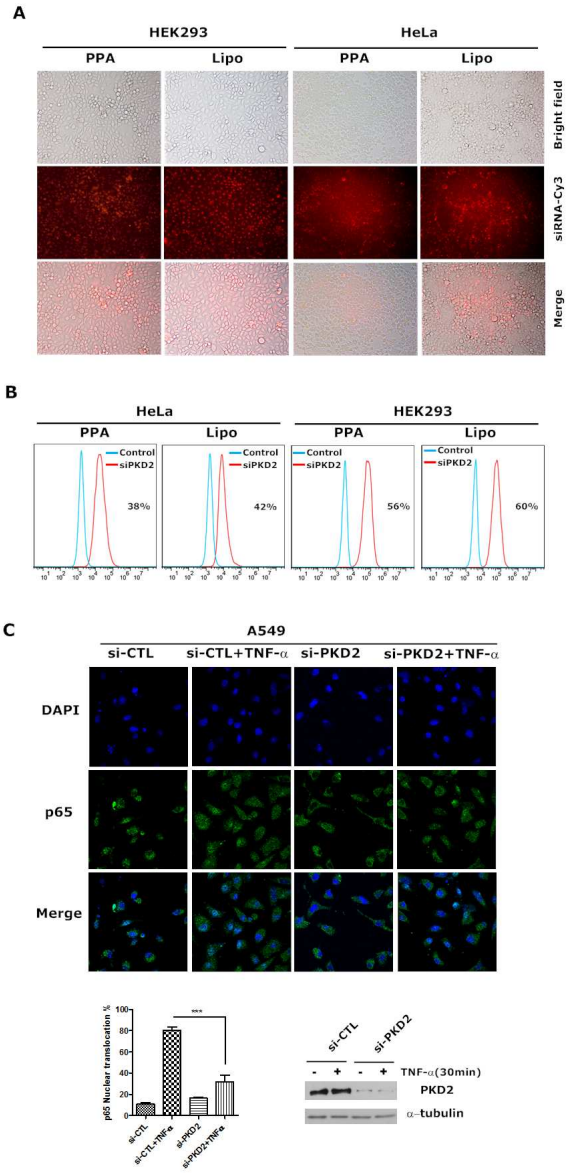


Figure 7 Transfection efficiency of siRNA encapsulated with PPA in different cells. (A) Silencing of endogenous PKD2 protein by transient siRNA/PPA and siRNA/Lipo transfection in HeLa and HEK293 cells, respectively. (B) FACS was performed to quantify the transfection efficiency in HEK293 and Hela cells using PPA and Lipofectamine ;(C) Immunofluorescence of NF-κB p65 localization in A549 cells, indicating that knockdown of PKD2 significantly inhibited downstream of p65 nuclear translocation induced by TNF-α(***p<0.001), Western blotting were applied to verify knockdown efficiency by siRNA/PPA transfection with or without TNF-α treatment in A549 cancer cells.

We then performed immunofluorescence staining and confocal microscopy to explore whether knockdown of PKD2 by PPA transfection can reduce TNF-α-induced NF-κB p65 nuclear distribution, which is the critical characteristic of NF-κB activation of gene transcription [32, 36], in response to TNF-α treatment. As shown in Figure 7C,TNF-α stimulation significantly increased NF-κB p65 nuclear distribution in siCTL-PPA transfected A549 cells , while siRNA knockdown of PKD2 by si-PKD2 /PPA transfection remarkably reduced NF-κB p65 nuclear translocation induced by TNF-α treatment in A549 cells, suggesting that siRNA could also be encapsulated and delivered

into target cells effectively, as well as exhibition of silencing function in physiological condition.

In order to evaluate the siRNA silencing with PPA transfection in different cells, we also transfected with siCTL or siRNA of PKD2 into Hela cells, after 60h, the transfected cells were treated with TNF- α for 30 min, confocal microscopy showed that similar results of NF-κB p65 nuclear translocation can be found in HeLa cells transfected by siRNA of PKD2 with or without TNF-α treatment(Figure 8A-B), Western blotting further confirmed that endogenous PKD2 could be effectively knockdown by siRNA transfection with PPA (Figure 8C). Taken together, these results indicated that our synthesized PPA not only can effectively delivery the plasmids into target cells, but also could form complex with siRNA and show knockdown efficiency as well as biological function of siRNA into the target cells.

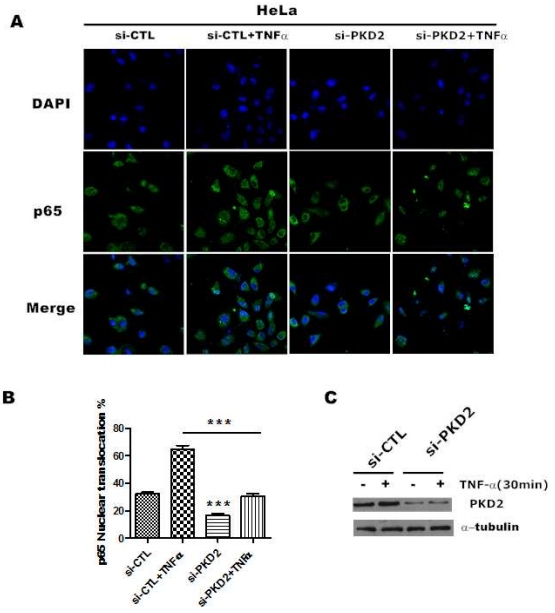


Figure 8 Transfection efficiency of siRNA-PKD2 and downstream of p65 nuclear translocation. (A) Immunofluorescence of NF-κB p65 localization in HeLa cells; (B) statistical analysis of p65 translocation significantly reduced in silencing of endogenous PKD2 by siRNA/PPA transfection with or without TNF-αtreatment in HeLa cells;(C) Western blotting was performed to analyse the knockdown efficiency of siRNA transfected by PPA.

Conclusions

In this study, we have developed a novel cation polyphosphoramide (denoted as PPA) with amino moieties in the main chain by polycondensation of ethyl dichlorophosphate with N^1-(2-aminoethyl)-N^1-(1-methyl)-1,2-ethanediamine. The PPA could efficiently retard mobility of DNA at lower N/P ratio, and exhibited a decreased size and increased zeta potential. The cytotoxicity assay and *in vitro* gene transfection further revealed that PPA were non-toxic to different cells and showed higher transfection efficiency at N/P ratio (3:1). Moreover, this kind of synthesized PPA can effectively deliver more than one exogenous genes into target cells and overexpression of these gene could show functional role in cells. More importantly, the novel PPA could also encapsulate the siRNA into different cells and exhibited silencing the target gene as well as the signaling pathway in A549 cancer cells and HEK293 cells. This study provides a new insight into the design of efficient and biocompatible gene and siRNA carriers using cation polyphosphoramide with amino moieties in the main chain as scaffolds.

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Notes and references

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