articles

molecular pharmaceutics

Polymeric Nanoparticles Containing Conjugated Phospholipase A2 for Nonviral Gene Delivery

Huong T. Le, Gururaj A. Rao, Aaron C. Hirko,* and Jeffrey A. Hughes

Department of Pharmaceutics, College of Pharmacy, University of Florida, Gainesville, Florida 32610

Received August 6, 2009; Revised Manuscript Received May 10, 2010; Accepted May 11, 2010

Abstract: Polyethylenimine (PEI) was conjugated to phospholipase A_2 (PLA_2) in an effort to improve transfection efficiency. PLA_2 was conjugated to PEI using EDC as a coupling reagent. The activity of enzyme in the conjugate was measured. DNA condensation ability of the conjugate to polymer was determined. The resultant nanoparticles were characterized by dynamic and electrophoretic light scattering. Two reporter genes were used to evaluate transfection efficiency in human embryonic kidney (HEK293) and human hepatoma (HepG2) cell lines. Conjugate was shown to retain PLA_2 activity and its ability to condense plasmid DNA, resulting in nanoparticles of a similar size to native PEI. The results demonstrated at N/P ratios of 15 and 20 showed 13- and 8-fold increase in transfection efficiency, respectively, compared to the maximum transfection efficiency of PEI (N/P ratio of 5) in the whole range of N/P ratios tested, from 5 to 60 in HepG2 cells. Toxicity studies in HepG2 cells showed uncomplexed conjugate had similar toxicity as PEI, and when complexed with DNA the conjugate had a significantly reduced toxicity. The results clearly indicate the potential for this approach to improve efficiencies of nonviral gene delivery vectors.

Keywords: Polyethylenimine; PLA_2; nanoparticles; nonviral gene delivery

Introduction

The aim of gene therapy is to deliver DNA and express a specific therapeutic protein or RNA molecule at the right level, time and location in order to have a positive impact on a disease state.[1,2] There are a number of intracellular barriers that must be overcome in order to be successful. First there must be cell adhesion in the target cells and tissue. Next, membrane translocation and nuclear transport must be accomplished to allow the plasmid DNA into the cell so that efficient transcription and translation occurs.[3,4] Naked plasmid DNA does not easily pass through cell membranes. Therefore, a carrier or a vector that can deliver genes into the cell is required. There are two main groups of carriers that have been extensively studied, viral and nonviral vectors. Viral vectors have evolved highly efficient strategies for transferring genes into the nucleus for gene expression. Viral vectors do have limitations, in particular, their relatively small packaging capacity, safety concerns, and immunogenicity.[5] Attempts to overcome the drawbacks associated with viral vectors have led to the development of alternatives. One such approach is the use of nonviral gene delivery vectors, termed "self-assembled" systems, is based on modified cationic polymers that form spontaneous complexes with anionic

* Corresponding author. Mailing address: University of Florida, College of Pharmacy, Department of Pharmaceutics, 1600 SW Archer Rd., P3-20, Gainesville, FL 32610. Tel: 352-273-7866. Fax: 352-392-3557. Email: ahirko@ufl.edu.

(1) Yasuhiro, Terazaki; Shojiro, Yano; Kentaro, Yuge; Satoshi, Nagano; Mari, Fukunaga; Sheng Guo, Z.; Setsuro, Komiya; Kazuo, Shirouzu; Kosai, K.-i. An Optimal Therapeutic Expression Level Is Crucial for Suicide Gene Therapy for Hepatic Metastatic Cancer in Mice. *Hepatology* **2003**, *37* (1), 155–63.

(2) Goverdhana, S.; Puntel, M.; Xiong, W.; Zirger, J. M.; Barcia, C.; Curtin, J. F.; Soffer, E. B.; Mondkar, S.; King, G. D.; Hu, J.; Sciascia, S. A.; Candolfi, M.; Greengold, D. S.; Lowenstein, P. R.; Castro, M. G. Regulatable Gene Expression Systems for Gene Therapy Applications: Progress and Future Challenges. *Melecular Therapy* **2005**, *12*, 189–211.

(3) Cristiano, R. J. Viral and non-viral vectors for cancer gene therapy. *Anticancer research* **1998**, *18* (5A), 3241–5.

(4) Paul, D.; Robbinsa; Ghivizzania, S. C. Viral vectors for gene therapy. *Pharmacology & therapeutics* **1998**, *80* (1), 35–47.

(5) Bouard, D.; Alazard-Dany, N.; Cosset, F.-L. Viral vectors: from virology to transgene expression. *Br. J. Pharmacol.* **2008**, 1–13.

10.1021/mp900192p
Published on Web 05/11/2010

plasmid DNA.[6] Condensing DNA with this strategy results in relatively small and positively charged particles that interact and are internalized by cells. One of the most effective polymers that has been widely tested *in vivo* and *in vitro* is polyethylenimine (PEI).[7]

Efficiencies of PEI and other nonviral gene delivery systems still pale in comparison to those of viral vectors.[8] It has been reported that transduction of some viral vectors is dependent on phospholipase A_2 (PLA_2) activity of the viral coat.[9,10] For example, the VP1 capsid protein of AAV contains a conserved domain of about 40 amino acids demonstrating PLA_2 activity.[11] Phospholipase A_2 catalyzes the hydrolysis of phospholipids, the main component of biological cell membranes. Therefore, our hypothesis is that conjugation of PLA_2 to PEI can improve transfection efficiency. The present study describes the design and characterization of a nonviral vector conjugate containing PLA_2. PEI−PLA_2/DNA polyplexes are examined in terms of DNA condensation ability, size and charge of nanoparticles, PLA_2 activity, gene expression efficiency and cytotoxicity.

Experimental Section

Chemicals. EDC [or EDAC; 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride], commercial grade; bee venom phospholipase A_2 (PLA_2), salt-free, lyophilized powder, 600−2400 units/mg protein; 2,4′-dibromoacetophenone or 4′-bromophenacyl (4-BPB) and Sephadex G-25, dextran beads with a diameter of 20−50 μm were purchased from Sigma Aldrich, St. Louis, MO; branched poly(ethyleneimine) 25,000 Da (polymer molecular weight) was obtained from Polysciences, Inc., containing 25% of tertiary amine groups, 25% of primary amine groups, and 50% of secondary amine groups. $sPLA_2$ assay kit was purchased from Cayman Cheme; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 4′-bromophenacyl bromide (4-BPB) were purchased from Sigma Aldrich, St. Louis, MO. Methanol, HPCL grade, was obtained from Fisher Chemical.

Enzymatic Assay Preparation. In the $sPLA_2$ kit, $sPLA_2$ concentrated assay buffer was diluted 10 times to get final assay buffer [25 mM Tris-HCl (pH 7.5) containing 10 mM $CaCl_2$, 100 mM KCl, and 0.3 mM Triton X-100]. The 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), a detector, was reconstituted with HPLC-grade water to yield 10 mM DTNB in 0.4 M Tris-HCl (pH 8.0). Diheptanoyl Thio-PC substrate was reconstituted with diluted assay buffer. Bee venom $sPLA_2$ control was diluted 100 times from stock provided.

Plasmid DNA. Two reporter genes used to evaluate transfection efficiency of the complexes were plasmid pLC0888 encoding the luciferase reporter gene under the control of a CMV promoter[12] and a plasmid expressing green fluorescent protein, pGFP 1418.

Cell Culture. HEK293 (human embryonic kidney cells) and HepG2 (human hepatoma cells) originated from American Type Culture Collection (ATCC), Manassas, VA. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Inc.) containing 4.5 g/L glucose, L-glutamine, sodium pyruvate, 10% (v/v) of fetal bovine serum (FBS) and 1% (v/v) of penicillin/streptomycin.

Evaluation of PLA_2 Activity. The PLA_2 assay was performed in a 96-well plate. The reaction mixture consisted of 10 μL of DTNB, 5 μL of assay buffer and either 10 μL of sample or 10 μL of bee venom PLA_2 (as positive control wells). Blank reactions included 10 μL of DTNB and 15 μL of assay buffer. Just prior to measuring the absorbance, 200 μL of substrate solution was added to all of the wells and mixed with the reaction mixtures. Absorbance was read every minute at 405 nm using a Synergy HT, BIO-TEK microplate reader.

Enzyme Activity Calculation. Absorbance was plotted for seven minutes and linear regression was performed using Graph Pad Prism v 4.0. The enzyme activity was calculated as the following equation.

$$sPLA_2^{\text{activity}} = \frac{\Delta A_{405}}{10.0\ \text{mM}^{-1}} \times \frac{0.225\text{mL}}{0.01\ \text{mL}} \times \text{sdr} \qquad (1)$$

Units of $sPLA_2$ activity are μmol/min/mL. ΔA_{405} is the slope from regression analysis, and sdr is the sample dilution ratio.

Syntheses of PEI−PLA_2 Conjugate. The schematic representation of the synthesis procedure is showed in Figure 1. Basically, N-substituted carbodiimide (EDC) reacts with carboxylic acids which are abundant in PLA_2 to form a highly reactive, *O*-acylisourea intermediate. This active species then reacts with primary amine in PEI to form an amide bond which is stable. PLA_2 (MW $\approx$ 14.5 kDa) from a stock concentration of 1 mg/mL or 69 pmol/μL and branched PEI

(6) Eliyahu, H; Barenholz, Y; AJ., D. Polymers for DNA delivery. *Molecules (Basel, Switzerland)* **2005**, *10* (1), 34–64.
(7) Demeneix, B; JP., B. Polyethylenimine (PEI). *Advances in genetics* **2005**, *53*, 217–30.
(8) Oh, S.; Pluhar, G. E.; McNeil, E. A.; Kroeger, K. M.; Liu, C.; Castro, M. G.; Lowenstein, P. R.; Freese, A.; Ohlfest, J. R. Efficacy of nonviral gene transfer in the canine brain. *J Neurosurg* **2007**, *107* (1), 136–44.
(9) Zadori, Z.; Szelei, J.; Lacoste, M. C.; Li, Y.; Gariepy, S.; Raymond, P.; Allaire, M.; Nabi, I. R.; Tijssen, P. A viral phospholipase A2 is required for parvovirus infectivity. *Dev Cell* **2001**, *1* (2), 291–302.
(10) Allal, C.; Buisson-Brenac, C.; Marion, V.; Claudel-Renard, C.; Faraut, T.; Dal Monte, P.; Streblow, D.; Record, M.; Davignon, J. L. Human cytomegalovirus carries a cell-derived phospholipase A2 required for infectivity. *J Virol* **2004**, *78* (14), 7717–26.
(11) Florian, Sonntag; Svenja, Bleker; Barbara, Leuchs; Roger, Fischer; Kleinschmidt, J. A. Adeno-associated virus type 2 capsids with externalized VP1/VP2 trafficking domains are generated prior to passage through the cytoplasm and are maintained until uncoating occurs in the nucleus. *Journal of virology* **2006**, *80* (22), 11040–54.
(12) Li, S.; MacLaughlin, F. C.; Fewell, J. G.; Li, Y.; Mehta, V.; French, M. F.; Nordstrom, J. L.; Coleman, M.; Belagali, N. S.; Schwartz, R. J.; Smith, L. C. Increased level and duration of expression in muscle by co-expression of a transactivator using plasmid systems. *Gene Ther.* **1999**, *6* (12), 2005–11.

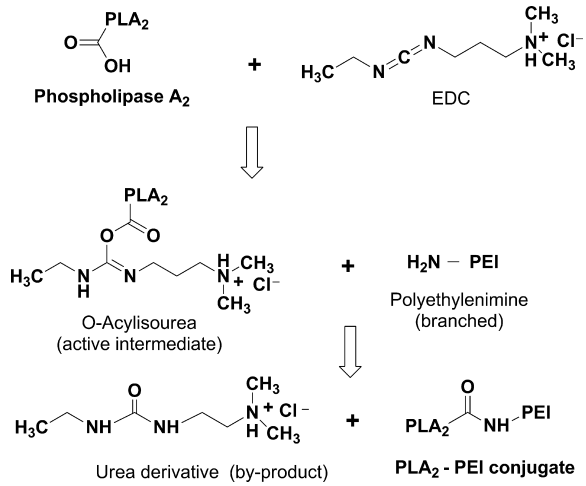

Figure 1. Schematic of the synthesis of conjugate.

(MW = 25 kDa) from a stock concentration of 2.15 mg/mL or 86 pmol/μL were mixed together with an excess amount of PEI at a molar ratio of 1:50 in HBS (7.5 mM Hepes and 150 mM NaCl) pH 6. The mixture was cooled on ice before addition of EDC (concentration of 500 pmol of solution in Hepes buffer pH 6) with the same molar ratio as PEI in HBS solution pH 6 to optimize the reaction. The reaction mixture was removed from ice after 5 min and stirred for 2 h at room temperature. Excess reagents (EDC and its byproduct (urea), small water-soluble molecules) were easily removed from the product using size exclusion chromatography. The product was purified by Sephadex G-25 column, 24 h hydrated in Hepes buffer pH 6, using HBS to elute conjugate from the byproduct, and the pure product was then aliquoted and stored at −20 °C for further studies.

Characterizations of PEI−PLA_2 Conjugate. Polyethylenimine quantification was performed using a modified method described by Ungaro.[13] A standard curve was prepared by adding 2.5 μL, 5.0 μL, 10 μL and 20 μL of 50 mM PEI to 1.0 mL of 1.0 mM copper(II) sulfate in water. Then the absorbance of the solutions at 285 nm was determined by a DU 800 UV−vis spectrometer. Sample absorbance was measured at the same wavelength in duplicate. The concentration of PEI in the sample was calculated using the standard curve.

Particle Size and Zeta Potential. Nanoparticles were made by mixing plasmid DNA and either PEI or conjugate in HBS solution pH ~6 at different N/P ratios between 10 and 60. The particle size and surface charge were measured with a 90 Plus particle size analyzer, Brookhaven Instruments Corporation, at 25 °C.

DNA Condensation. DNA condensation study was performed using an ethidium bromide (EtBr) exclusion assay.[14] The principle of this assay is based on the effect of concentration-dependent self-quenching of covalently bound fluorescence upon DNA collapse. EtBr intercalates between the DNA strands resulting in a dramatic fluorescence increase. When DNA is condensed by the cationic polymer, this intercalating fluorescent dye is displaced; uncomplexed DNA was used as the 100% control.

DNA Condensation Experiment. Plasmid DNA was mixed with various amounts of conjugate and PEI corresponding to N/P ratios of 1, 2, 4, 6, 8, 10, 20, 40, and 60 in Hepes buffer. After incubation for 15 min, EtBr was added and EtBr fluorescence was monitored at λ_{ex} = 485 nm, λ_{em} = 590 nm. The percentage of quenching for EtBr fluorescence was expressed as $((F - F_0)/(F_{max} - F_0)) \times 100$, where F is ethidium fluorescence of the complexes at different N/P ratios, F_0 is ethidium fluorescence mixed with Hepes buffer (blank), and F_{max} is ethidium fluorescence without DNA (control).

Transfection Studies. Cells were plated in 24-well plates at 10,000 cells/well and cultured for 24 h prior to transfection, by which time the adherent cells were ~80−90% confluent. Cells were overlaid with 200 μL of prewarmed serum-free medium containing 1% Pen/Strep before the addition of polyplexes, conjugate or native PEI complexes (corresponding 1 μg of DNA/well) in triplicates. Cells were exposed to the DNA particles in serum-free medium containing 1% Pen/Strep at 37 °C for 3 h after which the transfection medium containing DNA particles was replaced by fresh medium and incubation was continued for another 45 h. For studies in the presence of a PLA_2 inhibitor, polyplexes were prepared by mixing plasmid DNA with either conjugate or PEI (N/P of 20) in the presence of 1 mM 4′-bromophenacyl bromide (PLA_2 inhibitor).

Transfection Evaluation. Transfection efficiency was evaluated by luciferase activity *in vitro*. Medium from transfected cells was removed after 48 h and washed using 250 μL/well of phosphate-buffered saline (without Ca^{2+} or Mg^{2+}). Cells were then lysed by adding 150 μL of pH 7.8 luciferase lysis buffer (0.1 M potassium phosphate buffer, 0.1% Triton X-100, 1 mM dithiothreitol (DTT)) to each well and placed at −80 °C. Lysed cells were centrifuged at 14,000 rpm for 2 min at room temperature. 40 μL/sample was added to a white 96-well plate for measurement. 100 μL of assay buffer pH 7.8 (30 mM tricine, 3 mM adenosine triphosphate (ATP), 15 mM $MgSO_4$ and 10 mM DTT) was added to each well just prior to measuring.

GFP Analysis. The transfection efficiency was also evaluated with the HEK293 cell line using a plasmid containing a reporter gene encoding green fluorescence protein (GFP). The cells were incubated with various nanoparticle/DNA complexes for 3 h in serum free media

(13) Francesca, Ungaro; Giuseppe, De Rosa; Agnese, Miro; Fabiana, Quaglia. Spectrophotometric determination of polyethylenimine in the presence of an oligonucleotide for the characterisation of controlled release of formulations. *Journal of pharmaceutical and biomedical analysis* **2003**, *31*, 143–9.

(14) Trubetskoy, Vladimir S.; Slattum, Paul M.; Hagstrom, James E.; Wolff, Jon A.; Budker, V. G. Quantitative Assessment of DNA CondensationAnalytical. *Biochemistry* **1999**, *267*, 309–13.

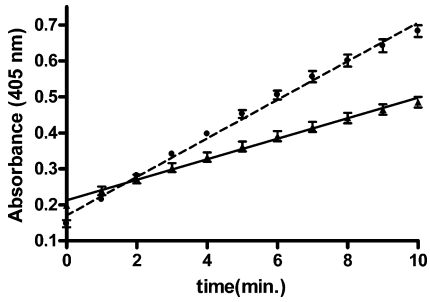

Figure 2. Phospholipase A_2 (PLA_2) activity before (●) and after (▲) conjugation to polyethylenimine (PEI).

then replaced with normal fresh media for endocytosis process and afterward, the transfection efficiency was qualitatively observed with a fluorescent microscope.

MTT Assay. Cells were cultured the same as described in Transfection Studies. Rapidly growing cells were harvested, counted, and inoculated at the appropriate concentrations into 96-well plates. After 24 h, treatments were applied in triplicate and cultures were incubated for 48 h at 37 °C. MTT (Sigma Aldrich) was prepared at 5 mg/mL in PBS (Dulbecco and Vogt formulation, without calcium or magnesium, Quality Biological, Gaithersburg, MD) and stored at −20 °C. 50 μL of MTT was added to the cells. The plate was protected from light, incubated at 37 °C for 4 h. After incubation, medium containing MTT was removed from each well, and 200 μL of 100% DMSO was added followed by 25 μL of glycine to all of the wells. After thorough mixing with a mechanical plate mixer, absorbance at 540 nm was measured with a Dynex Technologies model MRX microplate reader.

Results

Evaluation of Enzyme Activity before and after Conjugation to PEI. To evaluate if $sPLA_2$ retains its biological activity following conjugation a $sPLA_2$ activity assay was performed. The assay measures dynamic activity of the enzyme, as soon as the substrate (Diheptanoyl Thio-PC) is added, the enzyme will hydrolyze the thiol ester bond at the *sn-2* position yielding a yellow reaction product that is monitored by measuring the absorbance at 405 nm. Enzyme provided with the assay kit served as a positive control. The enzyme activity results are shown in Figure 2. Based on eq 1 enzyme activity was calculated to be 0.131 ± 0.003 μmol/(min·mL) prior to conjugation and 0.064 ± 0.003 μmol/(min·mL) following conjugation. As a control a 1:50 molar ratio of $sPLA_2$:PEI mixture was prepared without conjugation and the activity of the nonconjugated mixture was measured at 0.136 ± 0.006 μmol/(min·mL) (data not shown). The conjugate retained about 47.1% $sPLA_2$ activity consistent with a previous report when PLA_2 is bound

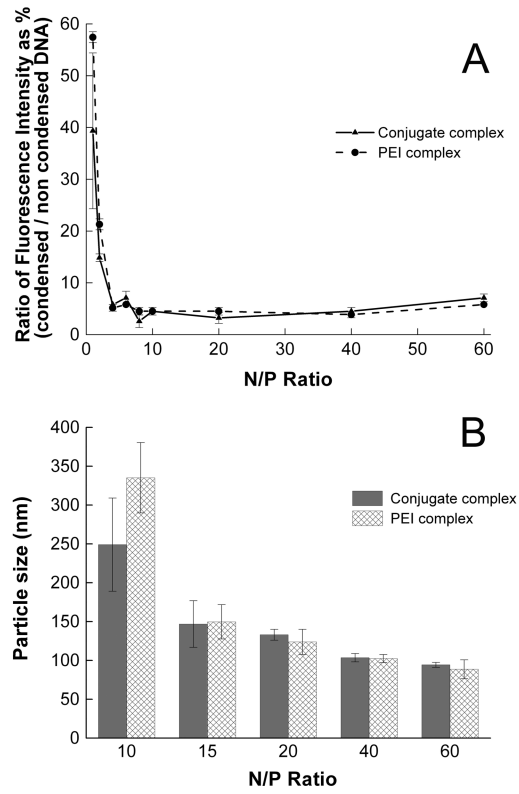

Figure 3. (A) Condensation-mediated fluorescence quenching. The fluorescence intensities different N/P ratios of either conjugate (▲) or PEI (●) interact with plasmid DNA were compared. Fluorescence intensity is plotted as a percentage ratio of condensed to noncondensed DNA. Uncomplexed DNA was used as the 100% control. (B) Size of polymeric nanoparticles formed by conjugate or PEI with DNA based on different N/P ratios.

through the carboxylic group using a modification of the carbodiimide method.[15]

DNA Condensation. The interaction of conjugate and PEI with plasmid DNA was tested using different N/P ratios ranging from 1 to 60. The condensation capacity of the conjugate and PEI were found to be similar (Figure 3A). As the N/P ratio is increased, the relative fluorescence decreased, indicating condensation of DNA. Both conjugate and PEI were able to bind to and condense DNA, more than 90% at an N/P ratio of 4. No significant differences were observed between conjugate and PEI in their ability to condense DNA.

Particle Sizing and Zeta Potential. The particle size and surface charge of nanoparticles formed when condensing DNA with conjugate or PEI was measured using dynamic light scattering. There were no significant differences detected in the particle size between conjugate and PEI (Figure 3B). As expected, particle size decreased to around 100 nm at higher N/P ratios. The surface charge remained positive, which is expected due to excess polymer in the complex

(15) Ferreira, J. P.; Sasisekharan, R.; Louie, O.; Langer, R. Influence of chemistry in immobilization of cobra venom phospholipase A2: implications as to mechanism. *Biochemistry* **1993**, *32* (32), 8098–102.

Table 1. Zeta Potential of PEI and Conjugate

N/P ratio	conjugate (mV)	PEI (mV)
10	41.43 ± 5.65	36.06 ± 4.34
15	32.96 ± 4.36	34.97 ± 5.55
20	33.97 ± 4.73	45.84 ± 4.39
40	30.44 ± 2.45	38.31 ± 5.65
60	33.87 ± 4.87	34.69 ± 6.34

(Table 1). Again, there were no differences detected between conjugate and PEI nanoparticles. In short, conjugation of PLA_2 to PEI does not change the particle size as well as the charge of the particle.

In Vitro **Transfection Studies.** Transfection efficiency was assessed using two different cell lines, HEK293 and HepG2, with a CMV driven luciferase plasmid (pLC0888). HEK293 cell lysates were assayed for luciferase activity after 48 h and the results are presented in Figure 4. The RLU/μg protein of PEI polyplex changes as N/P ratios are increased and drastically decreasing at N/P 60 (data not shown). Luciferase activity after transfection with PEI−PLA_2 conjugate complexes gradually increased as N/P increases. This indicates that higher concentrations of PLA_2 in the conjugate complexes lead to enhanced transfection efficiency. The transfection assay was done in triplicate for each N/P ratio of 5, 7, 10, 15, 20, and 40. In comparison with PEI, at low N/P ratios the conjugate showed lower transfection efficiency. However, at N/P ratio 40 the conjugate presents transfection efficiency significantly higher (~500 times) than PEI alone at the same N/P ratio.

Because transfection-efficiencies can be cell-line dependent, and HEK293 cells are known to be easily transfected, the complexes were also assayed using HepG2 cells. Again HepG2 cells were assayed for luciferase activity 48 h after transfection. As can be seen in Figure 5A the RLU/μg protein of the PEI polyplex gradually decreased as N/P increased, conversely the conjugate complexes either maintained or slightly increased the RLU/μg protein until N/P of 20 and then decreased at N/P 40. In comparison with PEI, at low N/P ratios the conjugate showed lower transfection efficiency, however, at N/P ratios 15, 20, and 40 the conjugate showed transfection efficiency remarkably higher (~115×, 1400×, and 2400× respectively) than PEI alone.

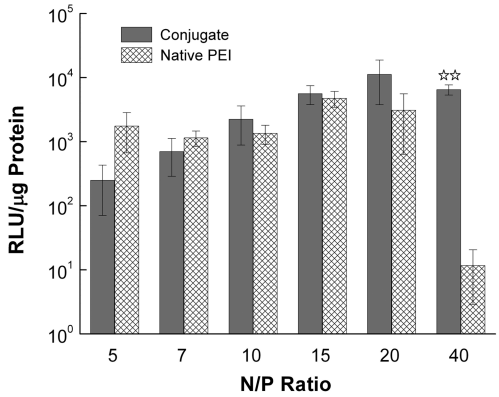

Figure 4. Transfection study of polyplexes on HEK293 cell line.

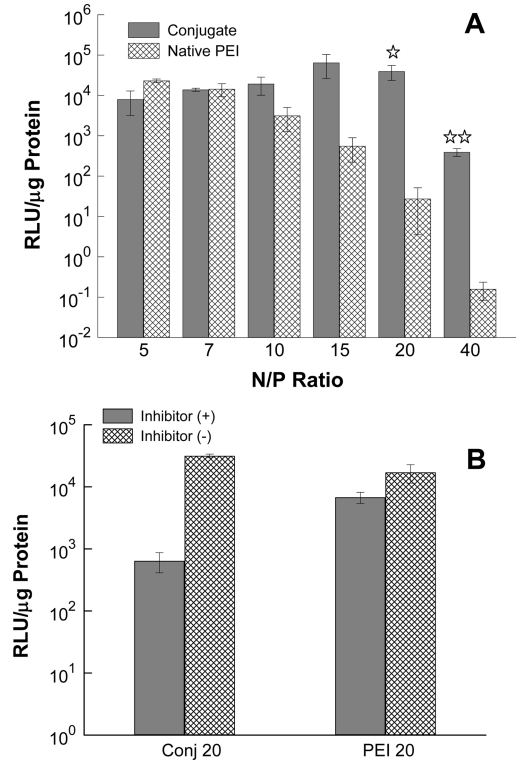

Figure 5. (A) Transfection study of polyplexes on HepG2cell line; and (B) Transfection of polyplexes in the presence of phospholipase A_2 inhibitor.

In order to examine whether the improved transfection efficiency was dependent on $sPLA_2$ activity HepG2 cells were transfected with both the conjugate at an N/P ratio of 20 in the presence of 1 mM 2,4′-dibromoacetophenone (4′-bromophenacyl bromide, a PLA_2 inhibitor). The presence of the PLA_2 inhibitor significantly reduced RLU/μg protein for the conjugate while having no significant effect on PEI's transfection efficiency (Figure 5B).

In Vitro **Transfection Study Using GFP as a Reporter Gene.** Transfection efficiency was also evaluated in HEK293 cells using a reporter gene encoding green fluorescence protein (GFP). Fluorescence microscopy revealed that the conjugate complex was able to mediate transfection of approximately 80% of HEK293 cells. The GFP^+ cells were distributed homogenously without any dead cells visible. The native PEI conjugate complex also shows transfection function but much less than that of the conjugate (~50% of the cells), GFP^+ cells were only seen in clusters and were located near the edge of the wells with many dying cells present (Figure 6).

Cytotoxicity Studies of Conjugate and Conjugate Complex vs PEI's. The cytotoxicity of the conjugate compared with PEI in the absence of plasmid DNA was investigated in HEK293 and HepG2 cells using an MTT assay. Cytotoxicity is increased as the concentration of PEI or the conjugate is increased in both HEK293 (Figure 7A) and HepG2 cells (Figure 7B).

The cytotoxicity of the conjugate in the presence of plasmid DNA was evaluated in HepG2 cells. Conjugate was

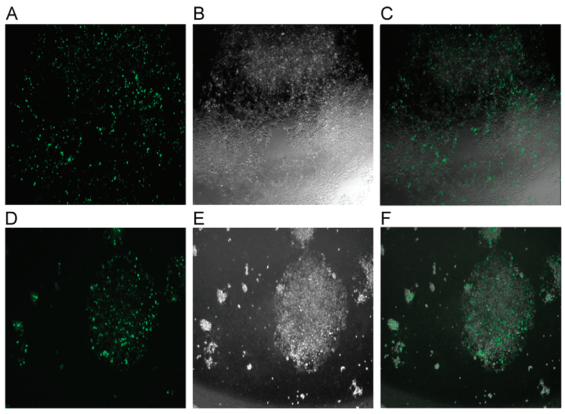

Figure 6. Transfection study of polyplexes of either conjugate (A, B, and C panels) or PEI (D, E, and F panels) complexed with a plasmid carrying GFP reporter gene into HEK293 cells at N/P ratio of 20. 5x magnification. Panels A and D are GFP flourecent images, B and E are bright field images and C and F are merged.

mixed with plasmid DNA at different N/P ratios to form nanoparticles. Cell viability decreased as N/P ratios increased with PEI complexes, in contrast to conjugate particles which maintained near 100% cell viability up to N/P ratio of 10 before showing decreases in cell viability similar to PEI at N/P of 15 and above (Figure 7C).

Discussion

This current report describes the novel use of an enzyme (Phosphlipase A_2) incorporated into a polymeric nonviral gene delivery system (PEI) with the aim of improving gene expression. We have demonstrated that conjugating PLA_2 to PEI does not affect the ability of PEI to condense DNA. The conjugation of PLA_2 also has no effect to the resultant nanoparticle's size or charge. At lower N/P ratios PLA_2 conjugated nanoparticles have similar transfection efficiencies as PEI nanoparticles,when normalized to total protein. However the inclusion of PLA_2 does significantly improves cell viability and at higher N/P ratios PLA_2 incorporation does significantly improve transfection efficiency.

There are a number of chemical and biophysical barriers that must be overcome in order to accomplish the efficient delivery and expression of nucleic acids. First the DNA must find and adhere to the target cells and tissue. Naked DNA is very unstable in the biological environment, it lacks cell specificity, and it is poorly taken up by cells. Condensation of DNA with cationic polymers forming polyplexes can protect DNA from degradation by nucleases.[16] Condensation can also result in a net positive charge allowing for interaction and adhesion with plasma membranes on target cells via electrostatic interaction. After adhesion of the polyplex occurs at the cell membrane of a target cell, a

(16) Pingoud, A.; Urbanke, C.; Alves, J.; Ehbrecht, H. J.; Zabeau, M.; Gualerzi, C. Effect of polyamines and basic proteins on cleavage of DNA by restriction endonucleases. *Biochemistry* **1984**, *23* (24), 5697–703.

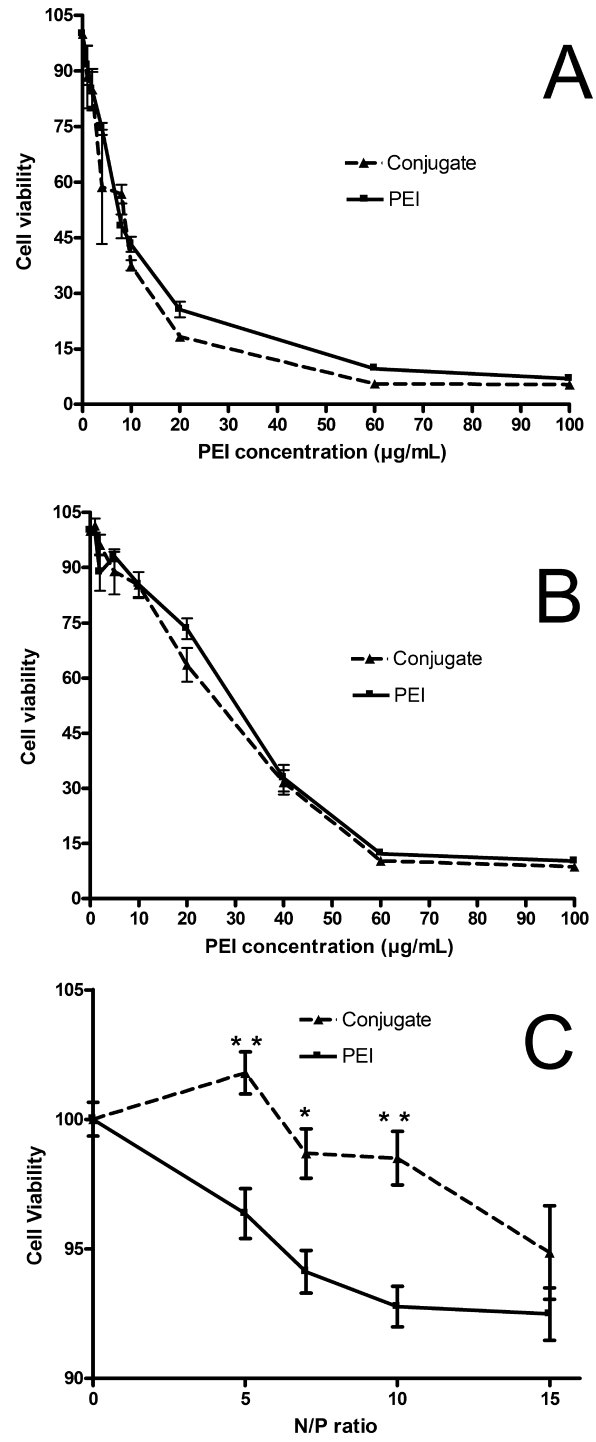

Figure 7. Cell viability of HEK293 cells (A), and HepG2 cells (B) after long-term exposure (48 h) to conjugate and PEI with no DNA present. Cell viability of HepG2 cells exposed to conjugate/DNA or PEI/DNA polyplexes (C) for 48 h and subsequently evaluated using a MTT assay (* $P < 0.05$, ** $P < 0.01$ by Bonferroni's multiple comparison test following 2-Way ANOVA).

number of barriers must be overcome for the DNA to enter the nucleus. The DNA must be taken into the cells and transported into the nucleus to allow for the DNA to be transcribed and translated.[3,4] The predominant mechanism of polyplex uptake into cells is endocytosis. Once polyplexes

become endocytosed they are localized within the endocytic vesicles or endosomes. Early endosomes can evolve into late endosomes that rapidly acidify to pH 5−6 due to an ATPase proton-pump enzyme in the vesicle membrane. The late endosomes fuse with lysosomes, which further acidify to pH and contain hydrolytic degradation enzymes. DNA trapped in the lysosomes will be degraded.[17−20] Therefore, a prompt escape from endosomes is required for the effective delivery of intact DNA into the cytoplasm. Once in the cytoplasm, transport to the nucleus and across the nuclear envelope must occur.

PEI has been shown to be very effective at the condensation and protection of plasmid DNA.[21] The branched poly amine structure gives PEI a large buffering capacity. According to Behr as the pH decreases from 7 to 5 the overall protonation of PEI increases from 20% to 40%.[22] This extraordinary buffering capacity of PEI results in what has been described as a "proton sponge".[23] This effect is thought to cause osmotic swelling in the late endosomes leading to rupture and release of the endosomal contents prior to fusion with the lysosomes.[24] These properties have resulted in making PEI one of the most efficient nonviral gene delivery vectors. However, PEI demonstrates toxicity and has been shown to induce apoptosis.[25]

Viruses have evolved a number of strategies to overcome the barriers to gene transfer. It has been discovered that a number of viruses including parvovirus have PLA_2 like domains contained within their viral coat.[9] These domains retain PLA_2 activity and this activity is necessary for infectivity.[26] Farr et al. showed that infectivity of PLA_2 null virions could be restored through either coinfection with PLA_2, or treatment with compounds such as PEI that disrupt the late endosome/early lysosome, suggesting that PLA_2 activity functions as a mechanism for endosomal escape in viruses.[27] Endosomal escape has been previously identified as a rate limiting step in nonviral gene transfer that could result in the largest improvement for transfection efficiency.[28] Recently the use of conjugated "fusogenic" peptides to polymeric gene delivery devices has shown success in increasing transfection efficiency.[29,30] Based on these observations and the fact that endosomal release is a major hurdle to gene delivery,[31] we hypothesized that incorporating the membrane-destabilizing agent PLA_2 into a PEI polyplex could assist the polyplexes release from the endosomal compartment. Our data however suggests that rather than improving transfection efficiency via enhanced endosomal escape, PLA_2 improves cell viability.

It has been reported that bee venom PLA_2 has its highest activity at $\sim$ pH 8.[32] This suggests that the lipolytic activity of our conjugated polyplex (pH $\sim$7.4) is most likely the highest at the cell membrane, the early endosome, or at the nuclear envelope.

The improvement observed in the toxicity profile of the PLA_2-PEI conjugated DNA nanoparticles could occur due to a number of reasons. The lipolytic activity of PLA_2 can hydrolyze phospholipids, the main component of cellular membranes, allowing for entry into the cell, bypassing the

(17) Wiethoff, C. M.; Middaugh, C. R. Barriers to nonviral gene delivery. *J. Pharm. Sci.* **2003**, *92* (2), 203–17.

(18) Brown, Maureen D.; Schatzlein, Andreas G.; Uchegbu, Ijeoma F. Gene delivery with synthetic (non-viral) carriers. *Int. J. Pharm.* **2001**, *229*, 1–21.

(19) Mattias, Belting; Staffan, Sandgren; Andres, Wittrup. Nuclear delivery of macromolecules: barriers and carriers. *Advanced Drug Delivery reviews* **2005**, *57*, 505–27.

(20) Pack, D. W.; Hoffman, A. S.; PUN, S.; Stayton, P. S. Degisn and development of polymers for gene delivery. *Nature reviews drug discovery* **2005**, *4*, 581–93.

(21) Godbey, W. T.; Barry, M. A.; Saggau, P.; Wu, K. K.; Mikos, A. G. Poly(ethylenimine)-mediated transfection: a new paradigm for gene delivery. *J Biomed Mater Res* **2000**, *51* (3), 321–8.

(22) Behr, J. The Proton Sponge: a Trick to Enter Cells the Viruses Did Not Exploit. *CHIMIA* **1997**, *51* (1−2), 34–36.

(23) Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci U S A* **1995**, *92* (16), 7297–301.

(24) Akinc, A.; Thomas, M.; Klibanov, A. M.; Langer, R. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. *J Gene Med* **2005**, *7* (5), 657–63.

(25) Hunter, A. C. Molecular hurdles in polyfectin design and mechanistic background to polycation induced cytotoxicity. *Adv Drug Deliv Rev* **2006**, *58* (14), 1523–31.

(26) Girod, A.; Wobus, C. E.; Zadori, Z.; Ried, M.; Leike, K.; Tijssen, P.; Kleinschmidt, J. A.; Hallek, M. The VP1 capsid protein of adeno-associated virus type 2 is carrying a phospholipase A2 domain required for virus infectivity. *J Gen Virol* **2002**, *83* (Pt 5), 973–8.

(27) Farr, G. A.; Zhang, L. G.; Tattersall, P. Parvoviral virions deploy a capsid-tethered lipolytic enzyme to breach the endosomal membrane during cell entry. *Proc Natl Acad Sci U S A* **2005**, *102* (47), 17148–53.

(28) Varga, C. M.; Tedford, N. C.; Thomas, M.; Klibanov, A. M.; Griffith, L. G.; Lauffenburger, D. A. Quantitative comparison of polyethylenimine formulations and adenoviral vectors in terms of intracellular gene delivery processes. *Gene Ther.* **2005**, *12* (13), 1023–32.

(29) Sasaki, K.; Kogure, K.; Chaki, S.; Nakamura, Y.; Moriguchi, R.; Hamada, H.; Danev, R.; Nagayama, K.; Futaki, S.; Harashima, H. An artificial virus-like nano carrier system: enhanced endosomal escape of nanoparticles via synergistic action of pH-sensitive fusogenic peptide derivatives. *Anal Bioanal Chem* **2008**, *391* (8), 2717–27.

(30) Moore, N. M.; Sheppard, C. L.; Sakiyama-Elbert, S. E. Characterization of a multifunctional PEG-based gene delivery system containing nuclear localization signals and endosomal escape peptides Acta Biomater 2008.

(31) Hoekstra, D.; Rejman, J.; Wasungu, L.; Shi, F.; Zuhorn, I. Gene delivery by cationic lipids: in and out of an endosome. *Biochem. Soc. Trans.* **2007**, *35* (Pt 1), 68–71.

(32) Kunze, H.; Bohn, E.; Eibl, H. On the mechanism of lysophospholipase activity of secretory phospholipase A2 (EC 3.1.1.4): deacylation of monoacylphosphoglycerides by intrinsic sn-1 specificity and pH-dependent acyl migration in combination with sn-2 specificity. *Biochim. Biophys. Acta* **1997**, *1346* (1), 86–92.

endocytic process. One would expect that toxicity should be increased if the cellular membrane is compromised. In fact under normal conditions, high concentrations of bee venom PLA_2 is toxic to cells. However at low concentrations (such as with the use of our conjugate complexes) and under conditions of cellular stress (as high levels of PEI would induce) such as hypoxia bee venom PLA_2 has been shown to be protective in a Ca^{2+} dependent manner.[33] This suggests that exogenous PLA_2 can have both beneficial and adverse effects on cells depending on its concentration. Our data clearly demonstrates that at tested N/P ratios, PLA_2 incorporated into PEI-DNA complexes significantly improves cell viability (Figure 7C). In fact it should be noted that at N/P ratios below 15 (a more commonly ratio for PEI transfection studies) incorporation of PLA_2 into PEI nanoparticles actually maintains cell viability at or above 100%.

One of the hydrolytic products of PLA_2 is lyso-phospholipids, which can act as surfactants increasing membrane permeability. This increase in membrane permeability can result in an earlier endosomal release than PEI without PLA_2. AAV is known to be released from the early to late endosome,[34,35] whereas PEI acting as a proton sponge in the late endosome/early lysosome results in osmotic swelling and eventual bursting of the vesicle.[36] PLA_2 may be coordinating an earlier release of the complex into the cytosol rather than complete bursting and release of other damaging endosomal components, resulting in less toxicity allowing for a more effective transfection reagent. The increase in membrane permeability could also be resulting in an easier entry across the nuclear membrane.

The other possible mechanism of how PLA_2 activity can improve transfection efficiency is that the hydrolytic products can have a direct effect on a number of cell signaling pathways. Products of PLA_2 activity such as diacylglycerol (DAG) and arachidonic acid have been shown to be critical players in numerous signal transduction pathways involved in inflammation, cell proliferation, differentiation, and apoptosis.[37−39] PLA_2 mediated modulation of these pathways may result in the improved cell viability we observed.

Future applications of the use of lipolytic enzymes for the delivery of drugs to cells are not limited to gene therapy. The delivery of protein drugs, siRNA, and other applications that may require high concentrations of polymers may benefit with the incorporation of lipolytic enzymes such as PLA_2 to the drug carriers resulting in improved viability, and delivery possibly increasing the effectiveness of the drug delivery.

Acknowledgment. The financial support of the National Institutes of Health (NIH Grant Nos. P01AG0485 and R21NS048782) is gratefully acknowledged, along with the gifts and equipment from the Department of Pharmaceutics, College of Pharmacy, University of Florida. We would also like to thank Dr. Wouter H. Driessen and Dr. Huan Tran for their technical contributions.

MP900192P

(33) Zager, R. A.; Schimpf, B. A.; Gmur, D. J.; Burke, T. J. Phospholipase A2 activity can protect renal tubules from oxygen deprivation injury. *Proc Natl Acad Sci U S A* **1993**, *90* (17), 8297–301.

(34) Bartlett, J. S.; Wilcher, R.; Samulski, R. J. Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. *J Virol* **2000**, *74* (6), 2777–85.

(35) Ding, W.; Zhang, L.; Yan, Z.; Engelhardt, J. F. Intracellular trafficking of adeno-associated viral vectors. *Gene Ther.* **2005**, *12* (11), 873–80.

(36) Godbey, W. T.; Wu, K. K.; Mikos, A. G. Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. *Proc Natl Acad Sci U S A* **1999**, *96* (9), 5177–81.

(37) Yagi, K.; Shirai, Y.; Hirai, M.; Sakai, N.; Saito, N. Phospholipase A2 products retain a neuron specific gamma isoform of PKC on the plasma membrane through the C1 domain--a molecular mechanism for sustained enzyme activity. *Neurochem. Int.* **2004**, *45* (1), 39–47.

(38) Farooqui, A. A.; Antony, P.; Ong, W. Y.; Horrocks, L. A.; Freysz, L. Retinoic acid-mediated phospholipase A2 signaling in the nucleus. *Brain Res Brain Res Rev* **2004**, *45* (3), 179–95.

(39) Farooqui, A. A.; Horrocks, L. A. Signaling and interplay mediated by phospholipases A2, C, and D in LA-N-1 cell nuclei. *Reprod Nutr Dev* **2005**, *45* (5), 613–31.