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pH-Responsive Biodegradable Assemblies Containing Tunable Phenyl Substituted Vinyl Ethers for Use as Efficient Gene Delivery Vehicles

Hee-Kwon Kim^{†,*}, David H. Thompson[‡], Ho Seong Jang[§], Yong Jin Chung^{||}, and Jeroen Van den Bossche[⊥]

[†]Division of Medicinal Chemistry, College of Pharmacy, The University of Texas at Austin, Austin, TX 78712, USA

[‡]Department of Chemistry, 560 Oval Dr., Purdue University, West Lafayette, Indiana 47907, USA

[§]Nano Materials Center, Korea Institute of Science and Technology, Seoul, 136-791, Republic of Korea

^{||}Chemical and Materials Engineering Department, California State Polytechnic University, Pomona, 3801 West Temple Ave., Pomona, CA 91768, USA

[⊥]Nanomedicine Laboratory, Centre for Drug Delivery Research, The School of Pharmacy, University of London, 29-39 Brunswick Square, London WC1N 1AX, UK

Abstract

Novel pH-responsive assemblies (PEG-lipid:DOPE liposomes) containing tunable and bifunctional phenyl substituted vinyl ether (PIVE) cross linkers were prepared. The assemblies consisted of 1, 2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), acidcleavable poly(ethylene glycol) (PEG)-conjugated lipids, pDNA and protamine sulfate (PS). The PIVE linkage was designed to hydrolyze under acidic conditions, and the hydrolysis studies of PEG-lipid compounds containing PIVE at pH 4.2, 5.4 and 7.4 indicated that the hydrolysis rates of PIVE linker were influenced by the substitution of electron withdrawing or electron donating groups on the phenyl ring. Acid-catalyzed hydrolysis of PIVE leads to destabilization of the acid labile PEG-PIVE-lipid:DOPE liposomes via dePEGylation, thereby triggering content release. Content release assays showed that dePEGylation was highly pH-dependent and correlated with the PIVE proton affinity of the phenyl group. These results indicated that the dePEGylative triggering based on a new pH-sensitive PIVE linkage can be controlled. *In vitro* transfection studies on the pH-responsive assemblies containing mPEG-(MeO-PIVE)-conjugated 1,3-dioctadecyl-*rac*-glycerol lipids (mPEG-(MeO-PIVE))-DOG showed higher transfection efficiency compared to that of polyethylenimine (PEI), a positive control, on HEK 293 and COS-7 cells. In addition, lower cytotoxicity of PEG-PIVE-lipid:DOPE liposomes/PS/DNA was observed in comparison to PEI. These results suggest that PEG-PIVE-lipid:DOPE liposomes can be considered as non-viral vehicles for drug and gene delivery applications.

Corresponding Author: hkkim717@utexas.edu; Tel: 512-475-9390; Fax: 512-471-4726.

ASSOCIATED CONTENT

Supporting Information

Additional kinetic data and figures (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

The Authors declare no competing financial interest.

Keywords

pH-Sensitive nanoparticles; Acid-cleavable poly(ethylene glycol)-lipids; Vinyl ether hydrolysis; Protamine sulfate; Gene delivery

Introduction

The construction of an ideal therapeutic delivery system (TDS) is a multidimensional challenge for the applications of gene therapy and chemotherapy in the fields of medicine and biotechnology.^{1–4} The TDS should exhibit a high and efficient uptake of the desired therapeutic moiety, shielding from the body's immune system and targeting to the disease site.⁵ In addition, to ensure that the TDS can exert its therapeutic action, a rapid release from the TDS will often be necessary while maintaining a low toxicity profile and decreasing side effects.^{6,7} Consequently, the scientific community is in search of mechanisms that allow for selective triggering of content release from the TDS at the disease site.

A popular way to achieve triggered release of therapeutics from a TDS is to utilize the pH-change that is inherently present in a variety of physiological and pathological processes such as endosomal processing, tumor growth and inflammation in acidic environments. When molecules enter cells through an endocytic pathway, a dramatic drop in pH from neutral to pH 5.9 – 6.0 in the lumen occurs within 2 minutes.^{8–10} The pH is further reduced from pH 6.0 to 5.0 during the progression from late endosomes to lysosomes. For example, the endosomal pH in African green monkey kidney (CV-1) cells and mouse peritoneal macrophages were reduced to 5.5 within 30 min and 4.7 within 3 h respectively.^{11,12} The endosomal KB cells and HepG2 cells have been shown to reach the pH values of 4.3 and 3.8 respectively.^{13,14} In addition, it has been known that the pH of tumors and inflammatory tissues are slightly acidic.^{15,16}

Ideally, a TDS should be stable under normal physiological conditions, but achieve rapid and efficient content release at its target sites. This requirement is the major challenge in the design of pH-responsive systems. One possibility to achieve this goal is through the construction of a TDS which is triggered through hydrolysis of pH-sensitive linkages. Subsequently, various pH-sensitive cross-linkages have been developed and the most common are ortho esters,^{17–19} cisaconityl,^{20,21} hydrazones,^{22,23} acetals,^{24,25} silyl ether,²⁶ and vinyl ethers^{27,28}

Liposomes are currently the most established drug delivery vehicles and bilayer membrane-based nanocarriers where the membrane is typically composed of phospholipids. Liposomes can be used for the delivery of both water-soluble and water-insoluble active pharmaceutical agents.²⁹ The physicochemical characteristics of liposomes can be easily controlled, and large scale production of these TDS is feasible.³⁰ High loadings of certain therapeutics also can be achieved through chemical gradients across the membrane bilayer via the remote loading technique.^{31–33} They can be easily formulated allowing for different well-characterized compositions, which can help enhance cellular permeation,^{34,35} enable active targeting,^{36–38} and promote intracellular trafficking and endosomal escape.^{39,40}

PEGylated liposomes have been shown to offer significant benefits over non PEGylated compositions such as improved biochemical and physical stabilities, a decreased immunogenic response, and an increase in blood circulation times.^{41,42} Consequently, researchers have attempted to construct pH-sensitive PEGylated liposomes to combine the benefits of triggering PEGylation with liposomal drug delivery.^{43–46}

The use of acid sensitive liposomes in the responsive tumor or endosomal environment has appeared as a promising strategy to increase the therapeutic effect in comparison to (non-triggered) conventional liposomal drug formulations due to prompt release and higher concentration of therapeutic agents accumulating at the target cell.⁴⁷

The strategy to create such acid sensitive liposomes is through a pH-sensitive stabilization of the lamellar phase of unsaturated phosphatidylethanolamine (PE) lipid such as DOPE.^{48–50} pH-Sensitive liposomes are prepared by incorporation of pH-sensitive cleavable PEG moieties into PE lipid. By fusion with the other phospholipids, curvature is increased, and the PEGylated lipid mixture is stabilized. This results in a lamellar phase, an L_{α} phase dispersion, that resists membrane fusion. Around a neutral pH such as pH 7.4, the PEGylated lipids will remain intact and in stabilization of the lamellar phase of the liposomes. However, acid-catalyzed dePEGylation of liposomes can be achieved through pH-sensitive linkages between the lipid and PEG at low pH. If a drop in the pH occurs, PEG chains were detached from liposomes by the cleavage mechanism of PEG moiety of acid sensitive PEGylated lipid as well as the protonation of headgroup of lipid, which makes lipid bilayer system destabilized by ensuing inversion of L_{α} phase to the inverted hexagonal-II (H_{II}) phase, thus resulting in promoting release of entrapped aqueous contents and membrane fusion with the adjacent membrane.

Thompson and co-workers have previously investigated this strategy through the synthesis of novel acid-labile PEG-conjugated vinyl ether lipids. These compounds have the ability to initiate acid-triggered content release from DOPE unilamellar liposomes. The structure-activity relationship for vinyl ether compounds was based on computational studies on the correlation between the proton affinity of vinyl ether molecules and the rate of hydrolysis.^{48,51}

The calcein release studies demonstrated that the rate of dePEGylation of DOPE was affected by the molar ratio of acid-labile PEG-lipids, molecular weight of the PEG and position of the pH sensitive vinyl ether bond with respect to the membrane interface.

Recently, a series of acid-sensitive PEG-lipids using a phenyl substituted vinyl ether (PIVE) linkage as a modulating substituent were synthesized. The pH-sensitivity of these materials was predicted using DFT calculation⁵² and confirmed using calcein release experiments. The main advantages of the developed chemistry are that it allows for an easy and orthogonal modification on the distal ends of the PIVE linker using a variety of methods and the pH-sensitivity can be predicted (see Figure 1). Consequently, it is possible to prepare a variety of materials with different pH-sensitive properties by incorporation of the PIVE linker.

Having previously established the advantages of the PIVE linkages, we wanted to further examine their utility for gene delivery (see Figure 2). The pH-sensitive linkages were prepared simply by addition of several substituents onto the phenyl ring close to vinyl ether to give more acid sensitivity, according to the concept of tunable and bifunctional PIVE cross linkers which was used in our previous study.⁵² Studies on cellular pDNA delivery have shown enhancement in the nuclear targeting and transfection efficiency with the addition of protamine.⁵³ Hence, protamine was chosen as a core condensing agent of plasmid DNA. For the purpose of this study, we attempted to combine the advantageous characteristics of the PIVE linker with protamine gene delivery. Thus, we constructed a new type of pH-responsive PIVE based assembly composed of mPEG-PIVE-lipid, DOPE, protamine sulfate (PS) and pDNA. After examination of the physicochemical characteristics of this novel gene delivery vector, we examined its efficacy *in vitro*.

EXPERIMENTAL SECTION

Materials

Unless otherwise stated, all chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO). Protamine sulfate was purchased from Sigma-Aldrich (St. Louis, MO). 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium (DOTMA) and mPEG-DOPE (PEG MW = 2000) were purchased from Avanti Polar lipids Inc. (Alabaster, AL). Agarose and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen (Carlsbad, CA). CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTT) was purchased from Promega (Madison, WI). The plasmid DNA (gWIZ GFP) was purchased from Genlantis (San Diego, CA) and the plasmid DNA (pCMV-Luciferase, encoding Photinus pyralis luciferase under control of the CMV promoter) was purchased from Promega (Madison, WI). The pDNAs were amplified in *E. coli* and prepared by the EndFree plasmid mega kit (Qiagen, Valencia, CA) according to the supplier's protocol.

Synthesis of mPEG-PIVE-Lipids

pH-Sensitive mPEG-PIVE-lipid conjugates, (1,3-bis(octadecyloxy)propan-2-yl(7-(((4-methoxy(ethyleneglycol[MW2000])carbamoyl)oxy) butoxy)-7-(4-methoxyphenyl)hept-6-en-2-y)carbamate (mPEG-(4-MeO-PIVE)-DOG), (1,3-bis(octadecyloxy)propan-2-yl(7-(((4-methoxy(ethyleneglycol[MW2000])carbamoyl)oxy) butoxy)-7-(2-methoxyphenyl)hept-6-en-2-y)carbamate (mPEG-(2-MeO-PIVE)-DOG), 1,3-bis(octadecyloxy)propan-2-yl(7-(((4-methoxy(ethyleneglycol[MW2000])carbamoyl)oxy) butoxy)-7-phenylhept-6-en-2-y)carbamate (mPEG-(H-PIVE)-DOG), 1,3-bis(octadecyloxy)propan-2-yl(7-(4-fluorophenyl)-7-(((4-methoxy(ethyleneglycol[MW2000])carbamoyl)oxy) butoxy)hept-6-en-2-y)carbamate (mPEG-(4-F-PIVE)-DOG), 1,3-bis(octadecyloxy)propan-2-yl(7-(2-fluorophenyl)-7-(((4-methoxy(ethyleneglycol[MW2000])carbamoyl)oxy) butoxy)hept-6-en-2-y)carbamate (mPEG-(2-F-PIVE)-DOG), and 1,3-bis(octadecyloxy)propan-2-yl(7-(4-(trifluoromethyl)phenyl)-7-(((4-methoxy(ethyleneglycol[MW2000])carbamoyl)oxy) butoxy)hept-6-en-2-y)carbamate (mPEG-(4-CF₃-PIVE)-DOG) were prepared according to the procedure reported by Kim et al.⁵²

Preparation and characterization of PS/DNA complex

A stock solution of protamine sulfate (10 mg/mL) was prepared using HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7.4). After dilution of protamine sulfate solution to the desired concentration, protamine sulfate/DNA complex was prepared by adding protamine in HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) to 100 μ L of pDNA solution (100 μ g/mL in HEPES buffer, 10 mM, pH 7.4) at several different N/P ratios. This formulation was incubated for 30 min at room temperature to allow for complexation after which they were immediately used for further experimentation.

The particle size of PS/DNA complex was measured by dynamic light scattering using a zetasizer (Nano ZS-90, Malvern, UK) and calculated using the manufacturer's supplied software.

The plasmid DNA condensation capacity of protamine with plasmid DNA under different N/P ratios was examined by 1% agarose gel electrophoresis using TAE buffer (242 g Tris, 57.1 mL glacial acetic acid, and 0.5 mM EDTA, pH 8.0) containing 0.5 μ g/mL ethidium bromide. Samples of each complex (100 ng) were prepared for gel electrophoresis. Protamine and plasmid DNA with different N/P ratios were prepared by vortexing the stock solutions at appropriate concentrations. These complexes were diluted using 10% serum in deionized water after which they were incubated at room temperature for 30 min. Each

complex sample was loaded on 1% agarose gel. A gel loading dye blue (New England BioLabs, MA) was added to each well, and agarose gel electrophoresis was carried out at a constant voltage of 80 for 50 min. The pDNA bands were visualized under a UV transilluminator at a wavelength of 365 nm.

Preparation of the mPEG-PIVE-lipid:DOPE liposomes loaded with PS/DNA

The stock solutions of pH-sensitive mPEG-PIVE-lipid conjugates and DOPE in chloroform were both prepared at 20 mg/mL concentrations. The lipid stock solutions (total 30 mg) in chloroform (1.5 mL) were combined at the desired ratios (2:98, 5:95, and 12:88 mPEG-PIVE-lipid conjugates/DOPE) and vortexed for 2 min. The chloroform was then evaporated using a flow of nitrogen gas, and a thin film of lipid was formed on the wall of a 10 mL vial after vacuum. Then, the lipid film was dispersed in 500 μ L of PS/DNA solution (20 mM HEPES, 150 mM NaCl, pH 7.4) and sonicated in a water bath at room temperature for 5 min. The resulting suspension was centrifuged and the resulting pellet was washed three times with deionized water (5 mL) to remove the uncoated liposomes. Finally, the resulting mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes were extruded through a 200 nm polycarbonate membrane filter. The particles were kept at 5 °C until further characterization was done.

Particle size and zeta potential measurement of the mPEG-PIVE-lipid:DOPE liposomes loaded with PS/DNA

The size and zeta potential of PS/DNA and mPEG-PIVE-lipid:DOPE liposome/PS/DNA complexes were determined using a zeta potential analyzer (Nano ZS-90, Malvern Instruments, UK). For the determination of the effective particle size and zeta potential, approximately 50 μ L of each mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes was diluted with 1 mL of 20 mM HEPES buffer (pH 7.4) to an appropriate concentration right before making the measurements. The measurements were carried out at room temperature and calculated using the manufacturer's supplied software. Experiments were run in triplicate.

Morphology of mPEG-PIVE-lipid:DOPE liposomes loaded with PS/DNA

The morphology and size of the mPEG-PIVE-lipid:DOPE liposomes loaded with PS/DNA were characterized using Tecnai F20 G² transmission electron microscope (FEI company, Hillsboro, OR) operating at 200 kV. Diluted mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes were dropped on a carbon-coated 400 mesh copper grid (3 mm in diameter) by pipette, and the excess solvent was evaporated before viewing it under TEM.

pDNA encapsulation efficiency

The pDNA encapsulation efficiency (EE) was determined from the ratio of the actual amount of pDNA encapsulated in mPEG-PIVE-lipid:DOPE liposomes to the initial amount of pDNA added for the fabrication of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes. The amount of encapsulated pDNA was obtained by measuring the difference between the initial amount of pDNA and the amount of pDNA present in the supernatant after centrifugation at 12000 rpm, 4 °C for 30 min. The supernatants were collected and analyzed using the PicoGreen assay kit (Invitrogen, Carlsbad, CA). All experiments were performed in triplicate.

Encapsulation efficiency(%) = (initial pDNA content added – free DNA content)/initial pDNA content added)×100

DNase digestion study

The digestion studies were performed according to the procedure reported by Gao et al.⁵³ In order to ensure the protection of mPEG-PIVE-lipid:DOPE liposomes and PS on pDNA, mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes (5 mg) were suspended with 2.5 U of DNase I (per μg DNA) in 10 mM Tris–HCl buffer containing 10 mM MgSO_4 (pH 8.0) for 30 min at 37 °C. After digestion, mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes were washed three times with 200 μL of fresh Tris–EDTA buffer. For extraction of DNA, fresh Tris–EDTA buffer with heparin (0.1%, w/v) was added to suspend mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes, and dichloromethane was used to solubilize mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes. The mixture was rotated end-over-end for 30 min at ambient temperature. The samples were centrifuged at 5000 rpm for 10 min, and supernatant was removed and analyzed by agarose gel electrophoresis.

Hydrolysis kinetics of mPEG-PIVE-lipid conjugates

For determination of the hydrolysis rate, pH-sensitive mPEG-PIVE-lipid conjugates (0.2 mg) were dissolved in 10 mL of 20 mM HEPES (pH 7.4), 20 mM acetate (pH 5.4) and 20 mM citrate (pH 4.2) and incubated at 37 °C. Aliquots (200 μL) were removed at each time point, and the hydrolysis rate was analyzed via HPLC. HPLC was performed at 40 °C on Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA) equipped with Eclipse XDB-C8 column (4.6×150 mm, Agilent technologies). Injection volume for HPLC was 40 μL , and the flowing gradient elution was performed with a flow rate of 0.7 mL/min: 0–20 min from 1:9 MeOH/ H_2O to 7:3 MeOH/ H_2O ; 20–35 min from 7:3 MeOH/ H_2O to 7:3 MeOH/ CH_3CN ; 35–50 min from 7:3 MeOH/ CH_3CN to 1:9 MeOH/ CH_3CN ; 50–60 min from 1:9 MeOH/ CH_3CN to 1:9 MeOH/ H_2O . The rate of hydrolysis was determined by monitoring the chromatographic peak area.

In vitro release assay

mPEG-PIVE-lipid:DOPE liposomes (10 mg) loaded with PS/DNA in microcentrifuge tubes were suspended in 1.5 mL of 20 mM HEPES (pH 7.4), 20 mM acetate (pH 5.4) and 20 mM citrate (pH 4.2). The suspension was placed in a shaking water bath at 37 °C at 120 rpm for the duration of the experiment. At each measurement point, the release medium was removed, and heparin (0.1%, w/v) was added to separate DNA from the PS/DNA complex. After the suspension solutions of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes were centrifuged at 12000 rpm for 10 min, the supernatant was collected for DNA release assay. The same volume of fresh medium was added to the mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes for resuspension and incubation in the fresh medium after rinsing. The collected sample was placed into a 96-well plate, and the amount of DNA released was measured by PicoGreen assay kit (Invitrogen, Carlsbad, CA). The amount of DNA released was calculated by comparing the pDNA content in the supernatant with the initial concentration of pDNA added. All measurements were performed in triplicate.

In vitro gene transfection

Luciferase activity was used to examine the transfection efficiency. Cells were seeded in 24-well plates at a density of 1×10^6 cells/well in 1 mL of complete DMEM medium supplement with 10% fetal bovine serum (FBS) at 37 °C, 5% CO_2 and 95% relative humidity and cultured one night before plasmid transfection. The culture medium was removed and replaced with serum free DMEM medium. mPEG-PIVE-lipid:DOPE liposomes loaded with PS/DNA was added in serum free DMEM medium and the cells were incubated with mPEG-PIVE-lipid:DOPE liposomes loaded with PS/DNA at 37 °C for 4 h in a humidified atmosphere with 5% CO_2 . After the medium was replaced by a fresh serum-supplemented culture medium, the cells were incubated with the transfection complexes at 37 °C for an

additional 20 h. The medium was aspirated and washed with PBS. The cells were lysed with lysis buffer, and the cell lysate was centrifuged at 12000 rpm for 10 min to pellet cell debris. The supernatant (25 μ L) was assayed for total luciferase activity using Luciferase Assay Reagent (Promega, Madison, WI), according to the standard protocol described in the manufacturer's manual. Luciferase activity was measured using luminometer (TD-20/20, Turner Designs, CA, USA). The transfection efficiency was expressed as Relative Light Units/mg protein (RLU/mg protein) after subtraction of background luciferase activity value from each sample value. RLU values were normalized to total cell protein. Also, gene transfections of PEI and lipofectamine were performed at N/P ratio of 6 as previously described.⁵⁴ All transfection experiments were performed in triplicate.

Cytotoxicity assay

The cytotoxicity of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes was evaluated by MTT assay. The cells were cultured in 200 mL DMEM supplemented with 10% FBS at 37 °C, 5% CO₂ and 95% relative humidity. The cells were seeded in a 96-well microtiter plate (Nunc, Wiesbaden, Germany) at a density of 1×10^5 cells/well and incubated for 24 h in culture medium. The culture medium was removed and replaced with serum free DMEM medium. mPEG-PIVE-lipid:DOPE liposomes loaded with PS/DNA was added in serum free DMEM medium, and the cells were incubated with the transfection complexes at 37 °C for 4 h in a humidified atmosphere with 5% CO₂. The culture medium was replaced with serum-supplemented culture medium, and the cells were incubated for 20 h. The media were replaced with fresh media, and 30 μ L of the MTT reagent was added to each well. The cells were incubated for an additional 2 h. The MTT solution was removed, and DMSO was added to dissolve the formazan crystals produced by living cells. Then, the absorbance was measured using a microplate reader (TECAN, Männedorf, Switzerland) at a wavelength of 490 nm. The cell viability (%) was compared with that of the untreated control cell in medium without mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes and calculated with $[A]_{\text{test}}/[A]_{\text{control}} \times 100\%$, where $[A]_{\text{test}}$ is the absorbance of the wells with mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes, and $[A]_{\text{control}}$ is the absorbance of the control wells. For PEI and lipofectamine, $[A]_{\text{test}}$ is the absorbance of the wells with PEI or lipofectamine, and $[A]_{\text{control}}$ is the absorbance of the control wells. All experiments were conducted in triplicate.

Statistical Analysis

All experiments were performed in triplicate, and the results were reported as means and standard deviations. Statistical analyses were performed using Student's t-test. The differences were considered significant for $p < 0.05$.

RESULTS AND DISCUSSION

Preparation of pH-sensitive PEG-lipid conjugates

In the present study, six pH-sensitive mPEG-PIVE-lipid conjugates, mPEG-(4-MeO-PIVE)-DOG, mPEG-(2-MeO-PIVE)-DOG, mPEG-(H-PIVE)-DOG, mPEG-(4-F-PIVE)-DOG, mPEG-(2-F-PIVE)-DOG, and mPEG-(4-CF₃-PIVE)-DOG containing different substituents on the phenyl rings were prepared following by a synthetic pathway previously described by Kim et al.⁵² The lipid anchors are coupled with PEG 2000 using a series of pH-sensitive heterobifunctional PIVE linkages. The aim of this work was to investigate whether electronic substituents of the phenyl ring would affect gene delivery constructs containing the mPEG-PIVE-lipids. The chemical structure of the mPEG-PIVE-lipids, which consists of mPEG, pH-sensitive PIVE linkage and a hydrophobic anchor, is shown in Figure 3. The PIVE linker has several advantages compared to other linkages. First, they are heterobifunctional, such that pharmacologically interesting compounds can be coupled to

one side of PIVE, and another useful molecule can be attached to the other side of PIVE. For example, in the mPEG-PIVE-lipid of the present study, the polymer [methoxypoly(ethylene glycol)] was linked to one side, and lipids were attached to the other side of PIVE. Second, they are tunable such that a series of molecules with PIVE can easily be prepared using a library approach. Each position of phenyl group can be modified by a number of functional groups to provide more or less acid-sensitivity to the vinyl ether bond.

Hydrolysis of mPEG-PIVE-lipid conjugates

Acid catalyzed hydrolysis of vinyl ethers occurs by protonation of the vinyl ether β -carbon, followed by rapid hydration of the transient and resonance-stabilized carbocation so that it can produce carbonyl and alcohol degradation products by decomposing the resulting heminal (see Figure 4). Because carbocation formation via the electrophilic addition of a proton to the vinyl ether is the rate-determining step of hydrolysis, the hydrolysis rate can be affected by modifying the electronic character of the α -substituent of the vinyl ether bond.⁴⁸ In order to investigate α -substituent effect of the phenyl substituted vinyl ethers, the hydrolysis kinetics of mPEG-PIVE-lipid compounds containing PIVE was evaluated at pH 4.2, 5.4 and 7.4 at 37 °C by monitoring both the disappearance of the starting materials and the appearance of the degradation products, 1-methoxypoly(ethylene glycol), MW 2000 (mPEG-OH), via HPLC-ELSD. The hydrolysis rate of each sample was examined by calculating the chromatographic peak areas of mPEG-PIVE-lipid compounds and mPEG-OH degradation product. Under all conditions studied, an increase in the peak area of the hydrolysis product, mPEG-OH, accompanied by a proportional decrease in the peak area of the starting material, mPEG-PIVE-lipid. The peak of mPEG-OH was verified by injection of a known commercial standard sample.

The study demonstrated the pH-dependent hydrolysis and the favorable pH-responsiveness to the acidic environment of the endosome, while maintaining its components at neutral pH. After 3 hours, the hydrolysis of mPEG-(H-PIVE)-DOG was 18% at pH 5.4 and 48% at pH 4.2, with half-lives of 9.92 and 3.48 hours respectively, whereas negligible hydrolysis was observed after 3 days at pH 7.4 (see Figure S1 in the Supporting Information).

To determine the influence of electronic effects, the hydrolysis rate of the PIVE linkage was examined by incorporation of electron donating groups (methoxy) and electron withdrawing groups (fluoro, trifluoromethyl) into the para-position of the phenyl ring and compared to the native PIVE (i.e. hydro). Relatively low hydrolysis was observed when the electron withdrawing group, fluoro, was added to the para-position of the phenyl ring (see Figure S1 in the Supporting Information). The hydrolysis rate of mPEG-(4-F-PIVE)-DOG at 37 °C for 3 hours was 16% at pH 5.4 and 45% at pH 4.2, and the half-lives were 11.1 and 3.3 hours, respectively. Remarkably slow hydrolysis was observed by incorporation of trifluoromethyl group, a strong withdrawing group, on the phenyl ring. At pH 5.4, 11 % of mPEG-(4-CF₃-PIVE)-DOG was hydrolyzed after 3 hours with a half life of 15.8 hours, and 31% was degraded at pH 4.2 ($t_{1/2}$ = 5.4 hours). On the other hand, as shown in the Figure 5, the hydrolysis of the mPEG-lipid conjugates was accelerated when the electron donating group, methoxy, was incorporated to the para-position of the phenyl ring. At pH 5.4, 35% of mPEG-(4-MeO-PIVE)-DOG was hydrolyzed after 3 hours with a half-life of 4.2 hours, and 99% was degraded at pH 4.2 ($t_{1/2}$ = 1.7 hours).

These results are in agreement with the hypothesis that the rate of hydrolysis of PIVE can be affected by varying the electron effects on the phenyl ring. Electron donating groups on the paraposition decreased the activation energy of the hydrolysis by stabilizing the transition state of the rate-determining step, and thus, significantly accelerated hydrolysis was observed.

Next, the hydrolysis rate of the PIVE linkage was examined by incorporation of substituents into the ortho-position of the phenyl ring to observe the effect of substituents' location. The substituents in the ortho-position of the phenyl ring appeared to affect the hydrolysis rate insignificantly. The hydrolysis rate for PIVE linkages containing the methoxy group and fluoro group on the ortho-position of the phenyl was not different from that of PIVE linkages with functional groups on the para-position (see Figure S1 in the Supporting Information).

Characterization of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes

In the present study, protamine was combined with pDNA as a condenser in order to perform gene delivery. Protamine, an arginine-rich peptide, is a safe and low molecular weight compound. It has been approved by the US FDA and is used as an antidote to heparin-induced anticoagulation.^{53,54} Protamine has four regions which are made up of amino acids, and are able to condense negatively charged DNA by electrostatic interaction between the positively charged arginine-rich domain and negatively charged DNA. It was reported that complexations of protamine with DNA can lead to higher enhanced transfection efficiency as compared to poly-L-lysine (PLL). In addition, it has been shown to help cellular pDNA delivery.^{56, 57}

In order to determine the optimal mass ratio, gel retardation assays were conducted to evaluate the complexation capacity of protamine with gWIZ GFP pDNA. In the present study, using a fixed quantity of DNA (100 ng/ μ L), a gradual quenching of the EtBr fluorescence from the DNA band was observed as the mass ratio of PS to DNA increased from 0.3/1 to 50/1 (N/P). From the gel electrophoresis results, more than 50% of pDNA was bound to protamine at a PS/DNA mass ratio of 0.3, and the unbound pDNA was no longer observed when PS/DNA mass ratio was 1.0 (see Figure 6). It indicated that complete retardation of the pDNA mobility was achieved at the PS/DNA mass ratio of approximately 1.0.

mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes were made up of mPEG-PIVE-lipid, DOPE, protamine sulfate (PS) and pDNA. These complexes were prepared via the thin film extrusion method using a mPEG-PIVE-lipid:DOPE ratio of 5:95 and analyzed for their particle size and surface charge. As shown in Table 1, all mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes had an average diameter of approximately 170 nm as measured by dynamic light scattering, and the observed zeta potential was found to be about 4.8 mV, which indicated that incorporation of PEG-PIVE-lipid decreased the surface charge of PS/pDNA via lipid coating and shielding of PEG. In this study, the encapsulation efficiency of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes was investigated because pDNA encapsulation efficiency was one of the important factors for the biological applications of delivery vehicles. pDNA encapsulation efficiencies of pDNA for the mPEG-PIVE-lipid:DOPE liposomes were in the range of 86 to 93% (table 1).

The structures of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes were evaluated by transmission electron microscopy. The shape of the 5:95 mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complex particles was spherical, as shown in Figure 7. It was found that the structures of these mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes remained unchanged for 3 days under a neutral condition (pH 7.4). One of the important features for gene delivery efficiency is the protection of pDNA. mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes were treated with DNase I to test whether these complexes could protect pDNA from nuclease digestion.⁵⁸ DNase I digestion treatment demonstrated that these complexes protected DNA from enzymatic degradation (see Figure S4 in the Supporting Information), while naked DNA was completely fragmented within 30 minutes of

incubation. These results indicated that DNA in mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes was stable towards enzymatic degradation.

Acid-triggered release from mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes

Content release studies from mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes were carried out using PicoGreen assay. mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes were incubated in different buffer solutions of pH 4.2, 5.4 and 7.4 at 37 °C and the time-dependent change in pDNA release was monitored. The pDNA release percentage at each time point was calculated by comparing the total amount of DNA in mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes to the amount of released pDNA.

From the kinetics of content release of 5:95 mPEG-(H-PIVE)-DOG:DOPE liposomes/PS/DNA complexes, the observed rate of pDNA release from liposomes/PS/DNA complexes with 5 mol% mPEG-(H-PIVE)-DOG after 8 hours was 45% at pH 4.2 (see figure S2 in the Supporting Information). Slower pDNA release was observed at pH 5.4 (19% over 8 hours). At pH 7.4, less than 10% release of pDNA was observed over 24 hours. These data show that faster pDNA release was observed at lower pH values, suggesting the kinetics of pDNA release from mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes was affected by the rates of dePEGylation and hydrolysis of PIVE under acidic conditions. The result was consistent with the reaction mechanism of vinyl ether hydrolysis. Hence, the hydrolysis rate of PIVE under acidic conditions has a direct impact on the pDNA release kinetics.

A variety of kinetics of pDNA release under acidic conditions was obtained from the mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes containing different functional groups on PIVE. The content release rates were directly affected by the type of PIVE linker used. As shown in the figure 8, the liposomes/PS/DNA complexes made up of 5:95 mPEG-(4-MeO-PIVE)-DOG:DOPE, which contained a methoxy group on the phenyl ring resulted in a 38% pDNA release at pH 5.4 within 8 hours (99% of the pDNA was released at pH 4.2 over 8 hours). However, slower pDNA release kinetics was obtained from the mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes with fluoro-substituted PIVE linkage in the phenyl ring (see table 2 and 3). At pH 5.4, only 14% of the pDNA was released from liposomes/PS/DNA complexes with 5 mol% mPEG-(F-PIVE)-DOG after 8 hours (33% of pDNA release occurred over 8 hours at pH 4.2). These results suggest that the observed hydrolysis rates affected by the electronic properties of the functional group of phenyl ring have a significant impact on the release kinetics of pDNA. Electron donating groups increased the hydrolysis rate and thus, increased the rate of dePEGylation, which accelerated the pDNA release significantly. On the other hand, incorporation of electron withdrawing groups on the phenyl ring led to the opposite result. Electron withdrawing groups decreased the rates of hydrolysis and dePEGylation. Therefore, a lower rate of pDNA release from the mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes containing fluoro group at the para-position of the phenyl ring was observed, and further reduced kinetics of pDNA release was observed from liposomes with trifluoromethyl group on the phenyl ring (see Figure S2 in the Supporting Information). The kinetics of pDNA release from the mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes with methoxy/fluoro-substituted PIVE linkage in the ortho-position of phenyl ring is similar to that of pDNA release from the complexes with functional group in the para-position of the phenyl ring (see Figure S2 in the Supporting Information). The release of DNA from mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complex nanocarriers can be correlated to the pH-sensitivity of the PIVE linkage. Consequently, we assume that dePEGylation results in collapse of the mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complex nanocarriers with DNA release due to phase inversion.

Fusogenic liposomes can be produced by blending DOPE with other lipids^{59,60} that aid the formation of a stable lamellar phase because the headgroups of lipid have negative charge which provides electrostatic repulsion to prohibit the lipid interbilayer interactions to convert it to hexagonal-II (H_{II}) at neutral pH. On the contrary, at low pH, the bilayer is destabilized due to the protonation of the headgroups of lipid, leading to membrane fusion with proximal bilayer membranes, formation of an inverted hexagonal II phase, and the release of entrapped aqueous contents.^{49,50} Thus, incorporation of higher mPEG-lipid content in mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes affects the kinetics of pDNA release. Our studies showed the effect of the amount of DOPE on the pDNA release from mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes (see figure S3 in the Supporting Information). Decreased rates of pDNA release occurred with mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes with 12 mol% mPEG-(H-PIVE)-DOG compared to liposomes/PS/DNA complexes with 5 mol% mPEG-(H-PIVE)-DOG due to the greater extent of hydrolysis required to destabilize the lamellar phase of DOPE liposomes containing higher molar ratios of mPEG-PIVE-lipid. However, mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes with 2 mol% mPEG-(H-PIVE)-DOG were observed to release more pDNA under the same condition. In contrast, minimal pDNA (less than 10%) was released from mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes after 2 days at pH 7.4. These results suggest that mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes are stable at physiological pH, making them suitable candidates for carrier systems for therapeutic delivery devices.

The structural consequences of PIVE linker hydrolysis on mPEG-PIVE-Lipid:DOPE liposomes were investigated using cryoTEM (see figure S6 in the Supporting Information). Incubated at pH 4.2 for 24 h, the structures had converted to dark small vesicle fragments with a diameter of 38–46 nm. It can be inferred that an L_{α} - H_{II} phase transition caused by acid-catalyzed dePEGylation of the mPEG-PIVE-lipid:DOPE liposome dispersion resulted in collapse of the mPEG-PIVE-lipid:DOPE liposomes into small H_{II} phase lipidic structures upon exposure to pH 4.2.⁵²

***In vitro* transfection study**

In vitro transfection studies were performed on HEK 293 and COS-7 cell lines with luciferase expression gene and compared with naked pDNA, a non-acidcleavable lipid (mPEG-DOPE), non-PEGylated lipid (*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium, DOTMA), PEI, and lipofectamine. For the control experiments with naked pDNA, only negligible transfection efficiency was observed. The result indicated that pDNA delivery efficiency is low in the absence of the carrier system. The transfection efficiency with PS/pDNA complex was slightly higher than that of naked pDNA, which was in agreement with previous reports.⁵⁷ As positive controls, commercially available PEI and lipofectamine were used. PEI and lipofectamine showed RLU in the range of $6.8 - 9.8 \times 10^5$ /mg protein in HEK 293 cells and $7.2 - 11.2 \times 10^5$ /mg protein in COS-7 cells.

Figure 9 shows the transfection efficiencies of a series of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes. When mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes were employed, we noticed a significant increase in the transfection efficacy, which was higher than when non-acid-cleavable lipid (mPEG-DOPE) and non-PEGylated lipid (DOTMA) were used. In addition, the transfection efficiency of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes with mPEG-(H-PIVE)-DOG was slightly increased in both HEK 293 and COS-7 cells compared to that of mPEG-(4-F-PIVE)-DOG:DOPE liposomes/PS/DNA complexes. And a significantly higher transfection efficiency of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes containing a methoxy group on the phenyl ring was observed. The complexes showed higher transfection efficiency than the positive controls, PEI and lipofectamine, in the HEK 293 and COS-7

cells. This increase in transfection efficiency of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes can be attributed to the combination of several factors: DOPE effect, release of pDNA from mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes and the stability of pDNA. DOPE has been widely used as one of the helper lipids. As a fusogenic lipid, it promotes cellular transfection by forming the H_{II} phase in the acidic environment of the endosome. It has been shown that transfection efficiency was significantly enhanced by incorporating DOPE into the formulation of lipoplexes.^{61–63} Also, the pDNA release was strongly related with the hydrolysis of the pH-sensitive properties of PIVE. Particularly, the electron donating methoxy group boosted the cleavage of the vinyl ether linkage and increased the transfection efficiency in the cell lines. In addition, it has been reported that the use of protamine enhances gene delivery.^{64,65} Condensation of pDNA with PS prevented destruction of pDNA during the cellular endocytic process by increasing the resistance of pDNA to enzymatic degradation. Our finding suggested that the stability of pDNA plays an important role in increasing the transfection efficiency as the transcriptional properties were retained by pDNA, which was protected by PS. Also, the stability of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes could be improved via encapsulation in the mixture of mPEG-lipid and DOPE.⁶⁴

In order to examine the serum compatibility of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes, transfection of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes was done in the medium with different serum concentrations. The result showed that gene transfection efficiency of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes at higher concentrations of serum media was higher than that of PEI (see Figure S7 in the Supporting Information). We believe that these synergetic interactions from mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes lead to the increased efficient delivery of transgenes.

***In vitro* cytotoxicity of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes**

A standard MTT assay was used to investigate the cytotoxicity of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes. As shown in Figure 10, after a 24-hour exposure of cells to mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes, the viabilities were analyzed to be approximately 90% for all of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes used. On the other hand, the use of equivalent amounts of PEI and lipofectamine led to 48% and 78% cytotoxicity. Our results indicated that the new type of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complex carriers enhances the transfection efficiency without causing significant cytotoxicity against HEK 293 cells. We have found that the cleavage of biodegradable PIVE linkage, which was used to prepare mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes, did not affect the cell viability but played a primary role in the content release at the acidic target site for efficient delivery. These low cytotoxicity results demonstrated that mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes are safer delivery vehicles with enhanced transfection efficiency than PEI and lipofectamine.

CONCLUSION

Using PIVE, a new class of acid-sensitive, tunable and bifunctional cross linkers, a novel pH-sensitive gene delivery system was constructed. PIVEs allow for using the intrinsic electronic effects on phenyl substituents of vinyl ethers and thus offer the control over the pH-sensitivity, which can be used for drug and gene delivery purposes. It was found that these linkers were degraded under acidic conditions, while they were stable at pH 7.4. The rate of acid-catalyzed hydrolysis could be controlled simply by changing electron donating/withdrawing groups of the phenyl ring. This makes it possible to design novel pH-responsive delivery carriers based on the concept of functional group selection. Here, the acid-sensitive PEG-lipid conjugates were used to create gene delivery vectors, which can be triggered by acid-catalyzed dePEGylation. The observed content release using acid-triggered

dePEGylation correlated well with the acidcatalyzed degradation of PIVE. This result confirmed that the rate of acid-catalyzed dePEGylation could be adjusted by controlling the hydrolysis of the cross linker. *In vitro* transfection efficiency of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes was influenced by introducing different functional groups onto the phenyl ring. The complex with mPEG-(4-MeO-PIVE)-DOG:DOPE loaded with PS/DNA was higher in transfection efficiency in HEK 293 and COS-7 cells but lower in cytotoxicity than the positive control, PEI. These results suggest that the mPEG-PIVE-lipid:DOPE liposomes can be a promising non-viral vehicle for the drug and gene deliveries that require more precisely controllable acid-triggering.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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$R_1 - R_5$: Electron donating/withdrawing substituents

Figure 1.

Chemical structure of phenyl substituted vinyl ether linkages

Figure 2. Schematic illustration of gene delivery pathways via acid-catalyzed dePEGylation from PEG-PIVE-lipids:DOPE liposomes through lamellar (L_{α}) to hexagonal (H_{II}) phase transition. The acidic environment triggers for hydrolysis of the linker, and subsequent dePEGylation facilitates the release of the cargo.

mPEG-(4-MeO-PIVE)-DOG : $R_1 = \text{OMe}$; $R_2 = \text{H}$
 mPEG-(2-MeO-PIVE)-DOG : $R_1 = \text{H}$; $R_2 = \text{OMe}$
 mPEG-(H-PIVE)-DOG : $R_1 = \text{H}$; $R_2 = \text{H}$
 mPEG-(4-F-PIVE)-DOG : $R_1 = \text{F}$; $R_2 = \text{H}$
 mPEG-(2-F-PIVE)-DOG : $R_1 = \text{H}$; $R_2 = \text{F}$
 mPEG-(4-CF₃-PIVE)-DOG : $R_1 = \text{CF}_3$; $R_2 = \text{H}$

Figure 3.
Chemical structure of mPEG-PIVE-lipid conjugates

Figure 4.
Acid-catalyzed hydrolysis mechanism pathway for mPEG-PIVE-lipid conjugates

Figure 5.

Hydrolysis rates of mPEG-(4-MeO-PIVE)-DOG at pH 4.2, pH 5.4 and pH 7.4. mPEG-PIVE-lipid conjugates were incubated at 37 °C. Aliquots were taken at each time point, and hydrolysis was analyzed via HPLC. The injection volume for HPLC was 40 μ L, and the flowing gradient elution was performed with a flow rate of 0.7 mL/min.

Figure 6.

Agarose gel electrophoresis retardation assay of PS/pDNA complexes at various N/P ratios. Lane1: pDNA as control. Lanes 2–7: PS/pDNA at N/P ratios of 0.3, 1.0, 6.0, 12, 25, and 50.

Figure 7. Transmission electron micrographs of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes. (a) mPEG-(4-MeO-PIVE)-DOG:DOPE liposomes/PS/DNA, (b) mPEG-(H-PIVE)-DOG:DOPE liposomes/PS/DNA, (c) mPEG-(4-F-PIVE)-DOG:DOPE liposomes/PS/DNA. White bars represent 200 nm.

Figure 8. pDNA release kinetics from 5:95 mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes at pH 4.2, pH 5.4 and pH 7.4. mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes were incubated at 37 °C. Aliquots were taken at each time point, and the pDNA release was measured.

Figure 9.
Transfection efficiency of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes.

Figure 10. Cytotoxicity of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes evaluated by MTT assay.

Table 1

Physicochemical characteristics of mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes.

Nanoparticles	Size (nm)	Polydispersity index	Zeta potential (mV)	Encapsulation efficiency (%)
PS/DNA	79.2 ± 6.78	0.430 ± 0.089	19.2 ± 1.53	
mPEG-(4-MeO-PIVE)-DOG:DOPE	171.8 ± 3.16	0.239 ± 0.069	4.8 ± 0.86	91.4 ± 2.1
mPEG-(2-MeO-PIVE)-DOG:DOPE	172.1 ± 2.56	0.199 ± 0.064	4.8 ± 0.82	92.5 ± 2.7
mPEG-(H-PIVE)-DOG:DOPE	169.7 ± 4.12	0.241 ± 0.041	4.7 ± 0.67	91.2 ± 3.6
mPEG-(4-F-PIVE)-DOG:DOPE	171.3 ± 3.76	0.240 ± 0.058	4.9 ± 0.83	86.5 ± 1.2
mPEG-(2-F-PIVE)-DOG:DOPE	170.8 ± 3.43	0.245 ± 0.082	4.8 ± 1.12	87.6 ± 1.6
mPEG-(4-CF ₃ -PIVE)-DOG:DOPE	172.2 ± 3.62	0.261 ± 0.078	4.8 ± 0.94	89.8 ± 1.8

Table 2

pDNA Release kinetics from mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes at pH 4.2.

Nanoparticles	120 min	480 min	1440 min
mPEG-(4-MeO-PIVE)-DOG:DOPE	38.3 ± 3.9	99.3 ± 5.1	100 ± 1.2
mPEG-(2-MeO-PIVE)-DOG:DOPE	38.9 ± 4.2	99.9 ± 3.2	100 ± 1.1
mPEG-(H-PIVE)-DOG:DOPE	16.5 ± 3.5	45.1 ± 4.3	78.9 ± 5.2
mPEG-(4-F-PIVE)-DOG:DOPE	12.2 ± 4.1	32.9 ± 2.3	70.0 ± 4.4
mPEG-(2-F-PIVE)-DOG:DOPE	11.9 ± 2.5	31.3 ± 3.2	69.8 ± 5.1
mPEG-(4-CF ₃ -PIVE)-DOG:DOPE	9.2 ± 1.3	26.9 ± 3.8	50.3 ± 3.5

Table 3

pDNA Release kinetics from mPEG-PIVE-lipid:DOPE liposomes/PS/DNA complexes at pH 5.4.

Nanoparticles	120 min	480 min	1440 min
mPEG-(4-MeO-PIVE)-DOG:DOPE	15.9 ± 3.7	37.6 ± 4.1	78.7 ± 5.1
mPEG-(2-MeO-PIVE)-DOG:DOPE	15.3 ± 2.5	38.5 ± 3.2	79.9 ± 4.1
mPEG-(H-PIVE)-DOG:DOPE	6.7 ± 0.7	18.6 ± 3.2	35.7 ± 3.2
mPEG-(4-F-PIVE)-DOG:DOPE	4.8 ± 2.5	13.6 ± 2.4	29.1 ± 4.9
mPEG-(2-F-PIVE)-DOG:DOPE	4.7 ± 1.8	12.9 ± 2.2	29.0 ± 3.2
mPEG-(4-CF ₃ -PIVE)-DOG:DOPE	3.9 ± 1.1	11.5 ± 1.8	21.7 ± 2.9