Gene Delivery Hot Paper

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Virus-Inspired Polymer for Efficient In Vitro and In Vivo Gene Delivery

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Abstract: *Clinical translation of nucleic acids drugs has been stunted by limited delivery options. Herein, we report a synthetic polymer designed to mimic viral mechanisms of delivery called VIPER (virus-inspired polymer for endosomal release). VIPER is composed of a polycation block for condensation of nucleic acids, and a pH-sensitive block for acid-triggered display of a lytic peptide to promote trafficking to the cell cytosol. VIPER shows superior efficiencies compared to commercial agents when delivering genes to multiple immortalized cell lines. Importantly, in murine models, VIPER facilitates effective gene transfer to solid tumors.*

The potentially transformative impact of nucleic acid-based drugs has not been clinically realized primarily owing to their considerable delivery challenges.[1] Two major classes of nucleic acid delivery vehicles, viral and non-viral systems, are used to package cargo and facilitate cell uptake, but both have their respective limitations. Viral vectors have immunogenicity and safety concerns and are expensive to manufacture.[2] Non-viral vectors such as synthetic polymers have improved cost and safety profiles but are generally orders of magnitude less efficient at gene transfer compared to their viral counterparts.[3]

Several studies investigating the intracellular trafficking of polymeric carriers have identified endosomal release to be the rate-limiting step in transfection of mitotic cells; if egress does not occur, endosomal contents are generally routed for lysosomal degradation.[4] Therefore, synthetic polymers have been designed to enhance endosomal release through mechanisms such as buffering in acidic pH (known as the proton sponge effect) and incorporation of membrane-active peptides and alkylated carboxylic acid.[5] These approaches typically work well in cultured cells but may not translate easily for in vivo applications. Endosomal buffering by the proton sponge effect requires significant accumulated polymer concentrations that may be difficult to achieve in vivo. Meanwhile, membrane-active peptides need to be shielded until reaching the endosome to minimize cell membrane disruption that results in off-site toxicity.

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In this work, we report a synthetic polymer that mimics the mechanism of efficient endosomal escape employed by adenovirus (Ad). In intact Ad, protein VI, the major protein involved in endosomal release,[6] is masked by a metastable viral capsid.[7] After cell-binding and internalization, the capsid rearranges in response to cellular cues. This conformational change in the capsid exposes the membrane-lytic protein VI, which then interacts with and destabilizes the endosomal membrane to trigger virus release into the cell cytosol.[8]

Inspired by the efficient intracellular trafficking of Ad, we designed a self-assembling synthetic polymer that contains a hidden membrane-lytic peptide at neutral pH. This polymer undergoes a conformational change in acidic environments, such as the early endosome, to unveil the peptide for selective endosomal membrane disruption. The peptide employed is melittin, which can insert into lipid membranes and induce pore formation.[9] Melittin has been conjugated to various cationic polymers and shown to enhance gene transfer ability by promoting endosomal release from the vectors.[10] We demonstrate that the melittin-containing polymer, VIPER, has selective membrane-lytic activity at acidic pH. VIPER effectively packages plasmid DNA for delivery into a variety of mammalian cells with efficiencies higher than commercial agents. Finally, we show that VIPER can be used for efficient in vivo gene transfer into tumors, overcoming the in vitro/in vivo disconnects observed for many polycation gene delivery systems.

In this work, we employed reversible addition–fragmentation chain transfer (RAFT) polymerization[11] to synthesize VIPER, which is composed of a hydrophilic cationic block for nucleic acid loading, and a pH-sensitive block for triggered display of a membrane-lytic peptide (Figure 1 A). The hydrophilic cationic block, poly(oligo(ethylene glycol) monomethyl ether methacrylate)-*co*-poly(2-(dimethylamino)ethyl methacrylate) (p(OEGMA-DMAEMA)), includes DMAEMA, widely used in polycations for nucleic acid delivery, and OEGMA, a hydrophilic monomer to provide colloidal stability.[12] The pH-sensitive block, poly(2-diisopropylaminoethyl methacrylate)-*co*-poly(pyridyl disulfide ethyl methacrylate) (p(DIPAMA-PDSEMA)), includes p(DIPAMA)), a pH-sensitive polymer that features a sharp phase transition from hydrophobic to hydrophilic at pH 6.3,[13] and PDSEMA monomers to enable further functionalization with thiol-containing peptides through disulfide exchange reactions. The successful synthesis of the copolymer, p($OEGMA_{11}$-$DMAEMA_{56}$)-*b*-p($DIPAMA_{33}$-$PDSEMA_1$) (control polymer used for further studies, denoted as CP), and VIPER (CP grafted with melittin) were characterized by ^{1}H NMR (Figure S1), GPC, and UV spectroscopy (Figure S2).

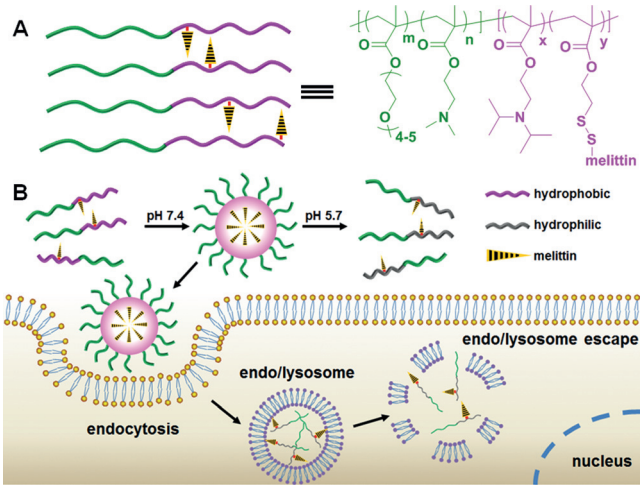

Figure 1. A) Chemical structure of VIPER (virus-inspired polymer for endosomal release). B) Illustration of VIPER-induced endo/lysosomal escape. At neutral pH, VIPER self-assembles into nanoparticles with melittin restricted to the pH-sensitive domain. After endocytosis by cells, the acidic endo/lysosome environment induces the hydrophilic phase transition of pDIPAMA, enabling melittin exposure, disruption of the endo/lysosomal membrane, and endo/lysosomal escape.

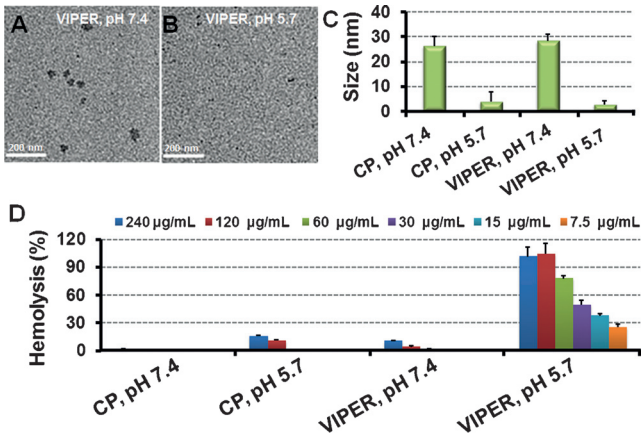

Figure 2. A) TEM image of VIPER at pH 7.4. B) TEM image of VIPER at pH 5.7. Scale bar: 200 nm. C) Average hydrodynamic diameter of polymer micelles at different pH values as determined by dynamic light scattering (DLS). D) Hemolysis activity of CP and VIPER at various concentrations and pH values. Data are shown as mean ± SD ($n = 3$).

VIPER was designed to self-assemble into micellar structures at physiological pH with melittin buried within the hydrophobic core. After endocytosis, the acidic endosomal environment triggers a hydrophilic transition of the pDIPAMA block, unveiling the melittin peptide, which can then facilitate endosomal release by disruption of the endo/lysosome membrane (Figure 1B).

To verify this hypothesized mechanism, the assembly behavior of VIPER at pH 7.4 and pH 5.7 was monitored by both transmission electron microscopy (TEM) imaging and dynamic light scattering (DLS) analysis. VIPER assembles into uniform spherical nanoparticles with diameter around 30 nm at pH 7.4 (Figures 2A and C for TEM and DLS, respectively). However, at pH 5.7, no assemblies were observed by either method, suggesting complete dissociation of nanoparticles at endosomal pH (Figures 2B and C). This data confirmed the pH-triggered phase transition of VIPER.

Next, a hemolysis study was conducted to evaluate the acid-triggered display of melittin. At pH 7.4, no significant cell lysis was observed even at VIPER concentrations as high as 240 $\mu g\,mL^{-1}$ after incubation with human red blood cells, confirming encapsulation and masking of melittin within the hydrophobic domain of the nanoparticles (Figure 2D). At pH 5.7, cell lysis was observed with a concentration-dependent profile: 50% lysis was achieved with only 30 $\mu g\,mL^{-1}$ VIPER and complete lysis occurred with 120 $\mu g\,mL^{-1}$ VIPER. Meanwhile, no significant membrane lysis was observed for CP in any of the testing conditions, indicating that membrane lysis is caused by the melittin rather than the polymer backbone.

The polymers were then complexed with plasmid DNA to form polyplexes for gene delivery. VIPER and CP efficiently packaged plasmid DNA, with complete condensation at an amine to phophate (N/P) ratio of 2 (Figure S3A). TEM imaging of VIPER and CP polyplexes (N/P = 5) revealed compact structures with relatively uniform spherical shape and diameters < 100 nm (Figures S3B and C). DLS measurements were consistent with the TEM images, and showed that particle sizes were similar between VIPER and CP complexes, and decreased with increasing N/P ratio (Figures S3D, S4). When formulated at N/P ≥ 3, the surface charge of both VIPER and CP complexes were positive (Figure S3E). Finally, a hemolysis study with CP/DNA and VIPER/DNA polyplexes confirmed that pH-sensitive membrane lysis was retained in the polyplex formulation (Figure S5). Cell lysis was observed with VIPER/DNA polyplexes at pH.5.7 but minimally at pH 7.4, and CP/DNA polyplexes were not membrane-active at either pH.

The lytic peptide melittin in VIPER is expected to induce the endo/lysosome escape of polyplexes. To evaluate endosomal release, we tracked both YOYO-1-labeled VIPER and CP polyplexes delivery to HeLa cells by triple fluorescence confocal microscopy after confirming that YOYO-1 fluorescence in polyplexes can be detected at both acidic and neutral pH (Figure S6). As depicted in Figure 3A, after 4 h incuba-

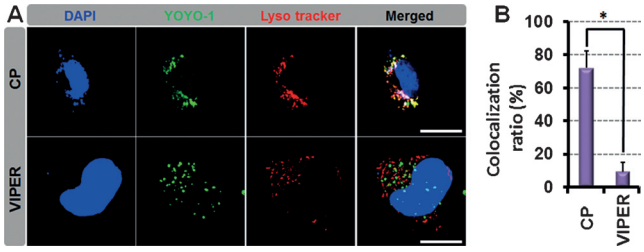

Figure 3. A) Confocal images of HeLa cells treated with polyplexes containing YOYO-1-labeled DNA for 4 h and stained with LysoTracker Red. DAPI (4′,6-diamidino-2-phenylindole, blue) was used to stain cell nuclei. Scale bar: 10 μm. B) Colocalization ratio of YOYO-1-DNA with LysoTracker-Red-stained endosomes. Results are presented as the mean of 15 individual cells. Data are shown as mean ± SD ($n = 15$; student's t test, $^*p < 0.001$).

tion, most of the green fluorescence (YOYO-1 DNA) from VIPER polyplexes was separated from the red fluorescence (LysoTracker Red), indicating efficient endo/lysosomal escape of VIPER/DNA polyplexes. Conversely, nearly complete colocalization (yellow) of green and red fluorescence was observed in cells treated with CP polyplexes. Consistent with the images shown in Figure 3B, the colocalization ratio of VIPER polyplexes with lysosomes was only 9.4%, compared with CP polyplexes (72.2%). Our previous work showed that bPEI polyplexes display similar intracellular distribution profiles as CP polyplexes, indicating endosomal trapping.[14] It is worthwhile to mention that there was no significant difference observed in cellular uptake efficiency between CP/DNA and VIPER/DNA complexes as determined by flow cytometry (Figure S7). Thus, VIPER polyplexes offer significantly improved endosomal release of nucleic acid cargo within mammalian cells compared to CP.

Next, the in vitro gene delivery efficiency of CP and VIPER polyplexes was tested in a panel of immortalized mammalian cell lines (HeLa and KB cervical carcinomas, A549 lung carcinoma, and Z310 choroidal epithelial cells) using a luciferase reporter plasmid. The polycation standard, branched PEI (bPEI, 25 kDa), and commercial transfection reagent, lipofectamine 2000 (LF), were used for comparison. The luciferase activity obtained by VIPER transfection was orders of magnitude higher than that by CP and bPEI in all of the tested cell lines (Figure S8–11). VIPER also mediated improved transfection efficiency compared to optimized LF formulations in HeLa, KB, and Z310 cells. VIPER polyplexes were well-tolerated; cell viability remained >90% in all of the cell types at all tested N/P ratios (Figure S8–11). The transfection experiment was then repeated using a reporter plasmid carrying the green fluorescent protein gene (GFP) to determine the percentage of transfected cells. The same transfection trend as the luciferase experiment was observed for all of the cell lines (Figure 4). VIPER transfected 13- to 60-fold more cells compared to CP and 11- to 46-fold more cells compared to bPEI, with efficiencies ranging from 36–77% GFP+ cells.

Gene therapy has been recognized as a possible treatment for various malignant tumors, and several polycation-based gene vectors have entered into different clinical phases.[15] To investigate the potential of VIPER as a gene vectors to treat cancer, we next evaluated in vivo gene transfer by direct delivery of polyplexes containing a luciferase plasmid to solid tumors in both KB and A549 xenograft tumor models. VIPER and bPEI polyplexes, which have membrane-lytic peptide (VIPER) and proton sponge (bPEI) mechanisms of endosomal escape, efficiently delivered the plasmid to KB tumors in contrast to CP, confirmed by their robust bioluminescence measurements in the tumor sites (Figure 5A).

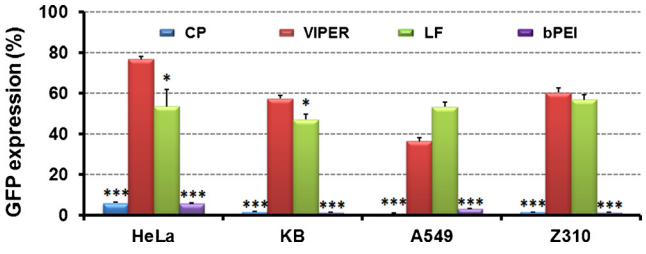

Figure 4. In vitro transfection of a GFP plasmid to various immortalized cell lines using optimized formulations. Transfections were conducted in serum-containing media except for LF, which was conducted in OptiMEM. All of the statistical analyses were performed compared to VIPER. Data are shown as mean ± SD ($n=3$; student's t test, $*p<0.05$, $***p<0.001$).

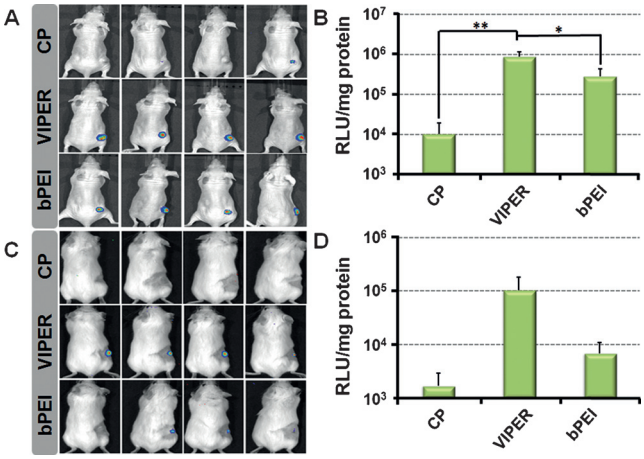

Figure 5. A) Bioluminescence images of KB-tumor-bearing mice treated by intratumoral injection with various polyplexes. B) Luciferase activity from excised KB tumor tissues of mice treated with polyplexes. C) Bioluminescence images of A549-tumor-bearing mice treated by intratumoral injection with various polyplexes. D) Luciferase activity from excised A549 tumor tissues of mice treated with polyplexes. Data are shown as mean ± SD ($n=4$; student's t test, $*p<0.05$, $**p<0.01$).

Tumors treated with VIPER polyplexes showed the highest luciferase activity, 3.1- and 82.5-fold higher than the bPEI- and CP polyplexes-treated groups, respectively (Figure 5B). VIPER polyplexes also outperformed both bPEI and CP polyplexes in intratumoral gene transfer to A549 xenograft tumors by 15.1-fold and 59.7-fold, respectively (Figure 5C and D). These results confirm that VIPER can efficiently mediate both in vitro and in vivo gene transfer to dividing cells.

Entrapment within endo/lysosomal vehicles is a major barrier in delivery of most macromolecular drugs with intracellular targets. For gene transfer, lysosomal nucleases, such as DNase II, degrade nucleic acids and compromise gene transfer efficiency.[16] To address this issue, diverse systems have been exploited, such as the proton sponge effect and the use of synthetic or virus-derived membrane-active peptides. However, previous reports have revealed that the "proton sponge effect" alone is not sufficient for endo/lysosomal escape.[17] Although the direct conjugation of lytic peptide with polycation carriers improves the transfection efficiency, significant cytotoxicity is typically associated with the peptides. For example, our previous work showed that the incorporation of either melittin or sHGP peptides to polycations results in cytotoxicity due to the exposure of the lytic peptide in physiological conditions and the destabilization of the plasma membrane.[18]

To address the issue of cytotoxicity versus transfection efficiency, the Wolff and Wagner groups developed masked melittin formulations, whereby melittin is reversibly blocked by maleic anhydride derivatives, rendering the peptide inactive at neutral pH and activated at by hydrolysis of the anhydride capping groups at acidic, endosomal pH.[19] The masked melittin materials mediated efficient delivery when co-injected with cholesterol-conjugated siRNA,[20] but polycations conjugated to melittin and siRNA showed liver toxicity when administered in vivo.[21] While effective, the anhydride protecting group is susceptible to hydrolysis even at physiological pH, which reduces the stability and shelf-life of the material. In contrast, the membrane-lytic peptide in VIPER is masked by hydrophobic encapsulation within the micellar core until exposure by a pH-triggered switch in the block copolymer. Thus, the pH-sensitive polymer acts as a responsive sheath to protect melittin. VIPER exhibits minimal membrane-lytic activity at pH 7.4, indicating efficient deactivation of melittin in neutral conditions, but becomes membrane-lytic in acidic environments (Figure 2D). Accordingly, VIPER polyplexes could also efficiently escape the endo/lysosomal compartments within 4 hours of internalization (Figure 3).

VIPER possesses other notable advances. First, the comonomer of OEGMA improves the stability of polyplexes, preventing salt-induced flocculation in physiological conditions, necessary for minimizing toxicity after in vivo administration (Figure S4). Second, the pDIPAMA block not only acts as a shielding part for melittin deactivation, but also as an efficient method to enhance the stability of polyplexes. Hydrophobic modification of polycations has been demonstrated to improve blood circulation time and transfection efficiency compared to parent polycations while reducing the cytotoxicity and promoting the overall biodegradability of gene carriers.[22] The VIPER/DNA polyplexes did not show obvious size changes in serum-reduced medium compared to distilled water, and maintained their dimensions for 20 h (Figure S4). Furthermore, intratumoral delivery studies demonstrated that VIPER polyplexes generated much higher gene expression in tumor sites compared to bPEI polyplexes (Figure 5). Thus, VIPER may provide a useful in vivo gene delivery platform for cancer therapy.

In summary, we report a virus-mimicking block copolymer that facilitates endosomal release, called VIPER. This polymer efficiently mediates both in vitro and in vivo gene transfer. The acid-triggered display of lytic peptide melittin effectively mediates endo/lysosomal escape of VIPER polyplexes, while masking melittin activity in extracellular conditions. VIPER therefore exhibits low cytotoxicity and potent endosomal escape properties and has strong potential as a delivery vehicle for macromolecular therapeutics.

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Keywords: DNA release · gene delivery · melittin · VIPER · virus-inspired polymers

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