

Acid-Degradable Polymer-Caged Lipoplex (PCL) Platform for siRNA Delivery: Facile Cellular Triggered Release of siRNA

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S Supporting Information

ABSTRACT: An acid-degradable polymer-caged lipoplex (PCL) platform consisting of a cationic lipoplex core and a biocompatible, pH-responsive polymer shell has been developed for the effective delivery of small interfering RNA (siRNA) through a combination of facile loading, rapid acid-triggered release, cellular internalization, and effective endosomal escape. In vitro testing of this degradable PCL delivery platform reveals ~45- and ~2.5-fold enhancement of enhanced green fluorescent protein knockdown in cancer cells in comparison to either free siRNA or siRNA-loaded non-acid-degradable lipoplex formulations, respectively.

Since its first application in mammalian cell gene knock-down,¹ small interfering RNA (siRNA) has garnered considerable interest both as a versatile biological probe and a highly potent therapeutic agent.^{2,3} The administration of siRNAs can induce enzyme-catalyzed degradation of their complementary mRNAs in diseased cells, thus disrupting the propagation of various diseases, such as cancers, diabetes, and virus infections.^{2,4} However, siRNAs are not ideal drugs from the conventional pharmacokinetic standpoint: They are highly susceptible to renal clearance⁴ and nuclease-mediated degradation in vivo.⁴ In addition, they also have poor cellular membrane penetration⁵ due to their large molecular sizes and high negative charges. Hence, for siRNAs to be successful as a therapy, they must be coupled to an effective delivery system that overcomes these limitations.

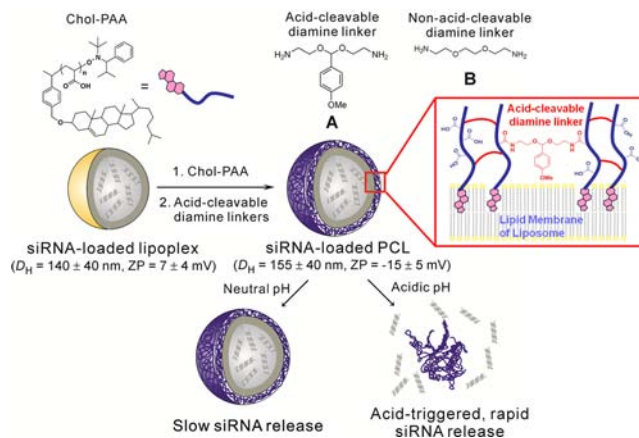
One of the most popular siRNA delivery platforms is based on cationic lipid formulations (lipoplexes)⁴ that allow for direct siRNA encapsulation without a need for modification of the fragile siRNA payload and/or conjugation to the delivery vehicle and their facile cellular uptake. However, their positive surface charges often cause unwanted toxicity, poor serum stability, and rapid uptake by reticuloendothelial system in vivo,^{2,4,6,7} limiting their therapeutic efficacy.

Coating cationic lipid-derived carriers with poly(ethylene glycol) (PEG), can reduce cytotoxicity caused by the stray binding of cationic lipids to cellular membrane⁸ and prevent their unstable aggregation with serum proteins.² Unfortunately, the PEG coating layer also hinders the interactions between the carrier and the endosomal membranes that is necessary for the former's escape from the endosome.⁹ As such, effective siRNA delivery into the cytosol by these platforms often requires a shedding of the PEG coating layer after cellular internalization.^{4,10} While remediating strategies based on electrostatic

interaction¹⁰ and acid-cleavable linkers^{11,12} have been reported for this task, these often require specially designed pH-responsive cationic lipids and/or additional covalent modifications to effect release and endosomal escape of the encapsulated siRNA.

Herein, we report a lipoplex-based, core-shell siRNA delivery platform (Scheme 1) that can be triggered to degrade

Scheme 1. Schematic Presentation of the Preparation of an Acid-Degradable siRNA-loaded PCL Nanocarrier and its Acid-Triggered Release of siRNAs. D_H and ZP Are Hydrodynamic Diameter and ζ Potential, Respectively



in acidic cellular endosomes, leading to selective siRNA release into the cytosol. Unmodified siRNA can be directly loaded into a lipoplex core composed of either a pH-responsive cationic lipid DODAP, whose tertiary ammonium head group has a 6.7 pK_a,¹³ or a non-pH-responsive cationic lipid DOTAP, with a quaternary ammonium head group, without the need for cumbersome covalent conjugation of siRNAs to the nanocarrier as necessary in other platforms.^{14,15} A biocompatible polymer shell comprising cholesterol-terminated poly(acrylic acid) (Chol-PAA) and acid-cleavable linkers was then constructed around the resulting lipoplex template to create an siRNA-loaded polymer-caged lipoplex (PCL). In acidic environments, such as cellular endosomes,¹⁶ this polymer network can shrink and induce the degradation of the PCL nanocarrier to accelerate siRNA release. This combined shrinking-degradation process is applied herein for the first time to acid-trigger

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siRNA release from cationic lipoplexes, a strategy that significantly differs from previously reported acid-degradable polymer-based siRNA delivery vehicles.^{17,18} The Chol-PAA anionic polymer component can additionally facilitate endosomal escape of the released siRNA into the cytosol, presumably by destabilizing the endosomal membrane.^{19,20} That both A-cross-linked DODAP and DOTAP formulations show similar in vitro release properties (cf. Figure 1 and Figure

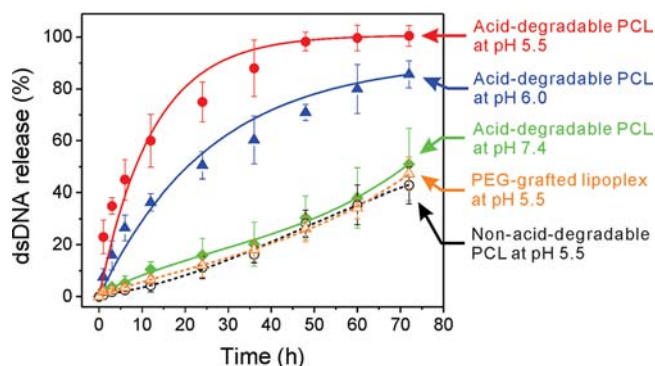


Figure 1. Time-dependent nucleic-acid-release profiles of the acid-degradable A-cross-linked DODAP PCL at different pHs. Also included for comparison are the time-dependent nucleic-acid-release profiles of the PEG-grafted DODAP lipoplex and the B-cross-linked DODAP-PCL, a non-acid-degradable analogue. All experiments were carried out at 37 °C.

S3a in the Supporting Information (SI)), suggests that the pH-responsive lipid DODAP does not play a major role in facilitating the acid-triggered release of encapsulated nucleic acids in our acid-degradable PCLs. As such, our [Chol-PAA + acid-cleavable linkers] core-shell formulation offers a major fabrication advantage over other pH-responsive cationic lipid nanocarriers:^{11,12,21} it sidesteps the need to incorporate specially designed pH-responsive cationic lipids necessary for the release and endosomal escape of siRNA from these other platforms.

To evaluate the effectiveness of our concept in siRNA transfection, we employed an eGFP-siRNA (sequence in the SI), one of the siRNAs designed to knockdown the mRNAs that express enhanced green fluorescent protein (EGFP).¹⁰ This unmodified siRNA was simply loaded into the cationic lipoplex by hydrating the appropriate lipid film with a buffered solution of eGFP-siRNA.¹⁰ The final loading of eGFP-siRNA in the cationic lipoplex is ~0.8 wt % siRNA/lipid (46% encapsulation efficiency), which is up to 3-fold higher than that shown for comparable formulations.¹⁰ Chol-PAA ($M_n = 5.4$ kDa and polydispersity index = 1.10) was then incorporated into the siRNA-loaded lipoplex at pH 7.4–8.0 using a “drop-in” method (Scheme 1, first step).²² As expected, the resulting polymer-grafted lipoplex (PGL) has a slightly increased diameter and a more negative ζ potential (ZP) as a result of Chol-PAA incorporation.²³

The grafted polymer shell of the siRNA-loaded PGL was then cross-linked with the acid-cleavable diamine linker A (SI, section S2)²⁴ to ~50% of the total number of the Chol-PAA’s carboxyl groups. The unchanged diameter of the resulting PCL and its homogeneous distribution indicates that these particles remain constant in size and do not aggregate in buffered saline solutions. The cross-linking of the PAA polymers increases the ZP of the PCL to a less-negative value, consistent with the

~50% conversion of the total carboxylic acid groups to amides. (Note: Please see Scheme 1 and Table S1 in the SI for hydrodynamic diameter and ZP data).

Because the acid-cleavable diamine linker A can be hydrolyzed readily under mildly acidic conditions (SI, Figure S2), we expect A-cross-linked PCLs to show enhanced nucleic acid-release profiles at acidic pHs. To quantify this enhanced release, we employed the more-stable, and less-expensive, double-stranded DNA (dsDNA) analogue (sequence in the SI) of eGFP-siRNA as the payload. As shown in Figure 1, our acid-degradable PCLs exhibit significantly enhanced nucleic acid-release rates at acidic pHs. At pH 5.5 and 37 °C, ~60% of the total amount of encapsulated dsDNA is released from the A-cross-linked PCLs within 12 h; quantitative release is observed within 48 h. In contrast, only small amounts of dsDNA are released at similar time points (~10% at 12 h and ~30% at 48 h) at pH 7.4 and 37 °C. Particle concentration data (SI, Figure S4) clearly show faster degradation of PCLs at pH 5.5 than at pH 7.4, suggesting that the accelerated release of dsDNA in acidic pH must have resulted from the degradation of these nanocarriers. This excellent acid-triggered release could also be applicable to the non-pH-responsive cationic lipid (DOTAP)-based PCL (SI, Figure S3a).

In our PCL formulation, the shell-localized network created by amide cross-linking the inserted Chol-PAA chains is essentially a poly[(AAc)-*co*-(AAM)] copolymer material that is well-known to “shrink” at acidic pH.²⁵ This chemo-mechanical response has been utilized to acid-trigger the release of encapsulated chemotherapeutics in macroscopic gels^{17,26} and anionic lipid-derived polymer-caged nanobins.^{22,27} It is also observed for our A-cross-linked PCLs formulations, in contrast to the lipoplex controls without PAA polymer and PEG-grafted lipoplex (SI, Figure S5). Despite a potential reduction in the “native shrinking” of the crosslinked polymer shell, attributable to interactions between the anionic PAA and the cationic-lipids, the combination of Chol-PAA and acid-cleavable linker A still confers rapid destabilization of the PCL membrane, eventually leading to degradation of the PCL nanoparticle. Such a combined shrinking-degradation process could allow researchers to choose from a wide range of available cationic lipids for efficient siRNA delivery. This is more advantageous than previously reported approaches,^{11,12,21,28} where one often must incorporate specially designed pH-responsive cationic lipids into lipoplexes for effective release and endosomal escape of siRNA.

As controls, we have also examined the release of encapsulated dsDNAs from PEG-grafted DODAP lipoplex, a traditional formulation with pH-responsive cationic lipids, and B-cross-linked DODAP PCLs, the non-acid-degradable analogue of our formulation,²⁹ both of which do not show as significant a release of dsDNA (Figure 1) as A-cross-linked PCLs under the same conditions. That the first control does not release nucleic acids at pH 5.5 is in line with reported observations that lipoplexes composed of pH-responsive cationic lipids do not significantly release encapsulated nucleic acids under acidic conditions unless they can make contact with anionic lipid membranes.⁴ That the second control also does not show significant release of nucleic acids under acidic conditions suggests that destabilization of the lipoplex core by the shrinking of the Chol-PAA polymer cage alone²² is not enough to release encapsulated nucleic acids from the PCL. These control experiments, together with the aforementioned fast degradation profile of our acid-degradable A-cross-linked

PCL, clearly support our hypothesis that a combination of acid-triggered “shrinking” and polymer shell degradation is critical for nucleic acid release from cationic lipid-derived A-cross-linked PCL nanocarriers.

To evaluate the biocompatibility of PCLs, human breast cancer (MDA-MB-231) and human cervical cancer (HeLa) cells were treated for 24 h at 37 °C with eGFP-siRNA-loaded PCLs. The treated cells exhibit ~100% viability over all tested siRNA concentrations (≤ 400 nM; ≤ 45.2 $\mu\text{g}/\text{mL}$ of cationic lipids). This is in stark contrast to the significant cytotoxicity shown by Lipofectamine 2000, one of the most popularly used cationic lipid-based formulations for gene and siRNA transfection (Figure 2). In our hands, the cells treated with the

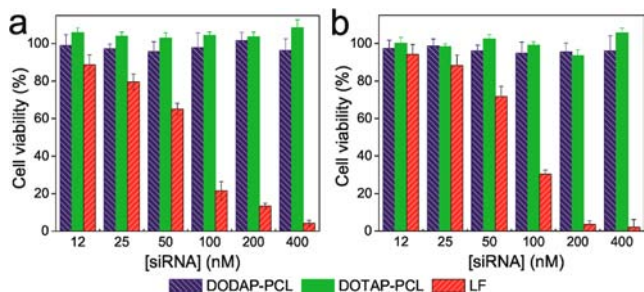


Figure 2. Dose-responsive cell viability of (a) MDA-MB-231 and (b) HeLa cells exposed to the eGFP-siRNA-loaded, A-cross-linked, acid-degradable DODAP-PCLs (left blue bar), DOTAP-PCLs (middle green bar), and eGFP-siRNA-loaded Lipofectamine 2000 (LF, right red bar) at 37 °C for 24 h. Error bars indicate the standard deviation of the mean with $n = 3$.

eGFP-siRNA-loaded Lipofectamine 2000 formulation shows <5% viability at 400 nM [siRNA] (20 $\mu\text{g}/\text{mL}$ of lipids). Thus, our acid-degradable PCL platform would be safer for clinical applications in comparison to currently used toxic cationic transfection agents.⁸

To evaluate EGFP knockdown efficiencies, MDA-MB-231 cells that have been engineered to stably express EGFPs were exposed to free eGFP-siRNA and four different eGFP-siRNA-loaded nanocarriers (PEG-grafted lipoplex, non-acid-degradable B-cross-linked PCL, and acid-degradable DOTAP- and DODAP-PCLs). The acid-degradable PCLs exhibit ~45% EGFP knockdown at 500 nM of siRNA, ~45- and ~2.5-fold enhancement over the free siRNA (~1% EGFP knockdown at 500 nM of eGFP-siRNA) and the non-acid-degradable nanocarriers (PEG-grafted lipoplexes and non-acid-degradable B-cross-linked PCLs; ~20% EGFP knockdown at 500 nM of siRNA), respectively (Figure 3a). Similar enhancements in EGFP knockdown by the acid-degradable PCLs were also observed for EGFP-expressing HeLa cells (Figure 3b). Indeed, confocal laser scanning microscopy (CLSM) imaging (SI, Figure S7) shows a visually clear decrease in the EGFP fluorescence signal intensity in both types of cells after treatment with the acid-degradable PCLs. These gene knockdown efficiencies are similar to those previously reported for PEG-coated lipoplexes¹⁰ and carbon nanotubes.¹⁵

While the gene knockdown efficiency by our acid-degradable A-cross-linked PCL increase over time (SI, Figure S8), its behavior in 95% serum does not change significantly from that in 10% serum-included media. This notable behavior is completely different from that of Lipofectamine 2000, whose gene knockdown efficiency dramatically dropped when being transitioned from 10% serum-included media into 95% serum

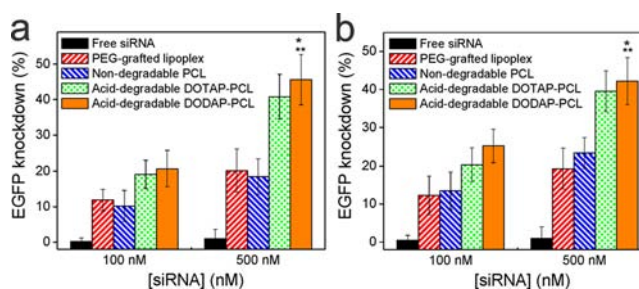


Figure 3. EGFP knockdown efficiency in (a) MDA-MB-231 and (b) HeLa cells by free eGFP-siRNA (black) and siRNA-loaded formulations (PEG-grafted lipoplex (red), non-acid-degradable PCL (blue), and acid-degradable DOTAP-siRNA (green) and DODAP-PCLs (orange)) at 100 and 500 nM of eGFP-siRNA. The data were assessed by flow cytometry. Error bars indicate the standard deviation of the mean with $n = 3$. * denotes $p < 0.001$ compared to free siRNAs. ** denotes $p < 0.01$ compared to siRNA-loaded PEG-grafted lipoplex and siRNA-loaded B-cross-linked PCL.

(SI, Figure S9) and points to a serum-independent advantage that our PCL may have over Lipofectamine and similar formulations.

The polyanionic Chol-PAA has been known to destabilize endosomal membranes and lead to endosomal escape of biomolecules.¹⁹ As such, we hypothesized that the Chol-PAA component of our PCLs could further facilitate the escape of nucleic acids from endosomes into cytosol after acid degradation, which facilitate their desorption from the shell. To verify this, MDA-MB-231 and HeLa cells were treated at 37 °C for 24 h with PCL formulations that have been loaded with Cy5-labeled dsDNA (Cy5-dsDNA) analogue of eGFP-siRNA as the payload. These cells were then treated with a LysoTracker Green dye to stain acidic endosomes/lysosomes. CLSM images of the cells that have been exposed to the dsDNA-loaded PCLs (Figure 4) show that Cy5-dsDNA (red-colored regions) have

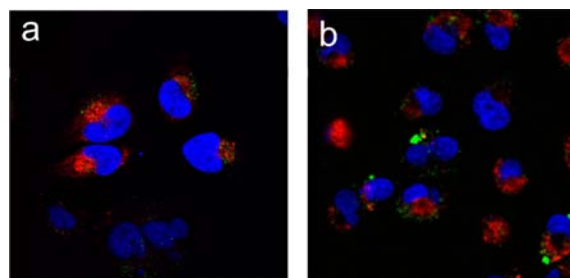


Figure 4. CLSM images illustrating the intracellular distribution of Cy5-dsDNA (red) and acidic endosomes/lysosomes stained with LysoTracker Green (green) in (a) HeLa and (b) MDA-MB-231 cells. The cells were treated with Cy5-dsDNA-encapsulated DODAP-PCLs (100 nM DNA) for 24 h at 37 °C. The nuclei were stained with Hoechst dye (blue).

spread throughout the cells with minimal overlap with the acidic endosomes/lysosomes (green-colored regions), indicating successful endosomal escape of Cy5-dsDNA and its localization in the cytosol. In contrast, CLSM images of MDA-MB-231 and HeLa cells that have been exposed to a Cy5-dsDNA-loaded PEG-grafted lipoplex formulation (SI, Figure S10) show more localized Cy5-dsDNA regions that significantly overlap (yellow-colored regions) with the acidic endosomes/lysosomes, indicating that there are more nucleic acids trapped inside the endosomes/lysosomes in these cells.

Together, these results strongly support our hypothesis that the Chol-PAA component of the PCL could indeed facilitate the release of nucleic acid from the trapped endosomal/lysosomal stage into the cytosol.

In conclusion, we have demonstrated an acid-degradable cationic lipid-based PCL core-shell system that exhibits excellent potential as an siRNA delivery platform. This outstanding biocompatible PCL platform can be easily fabricated from easily accessible components and the desired nucleic acids via a drop-in method. It shows high efficiencies in the knockdown of EGFP in cancer cells through rapid acid-triggered release, enhanced cellular internalization, and effective endosomal escape of unmodified siRNA. As a platform, it is more advantageous than Lipofectamine 2000 by being much less cytotoxic and can maintain the same gene-knockdown efficiency over a broad range of serum-containing media.

Moreover, our acid-degradable PCLs offer a major fabrication advantage over previously reported pH-responsive, PEG-coated cationic lipid-based siRNA nanocarriers^{11,12,21} in its broad applicability to many lipid formulations: The drop-in modification replaces the need for incorporating specially designed pH-responsive cationic lipids traditionally required for the effective release and endosomal escape of siRNA. In addition, the Chol-PAA-based shell of the PCLs has carboxylic acid groups that can be used as chemical handles for further conjugation with cancer-targeting ligands,²³ imaging probes,³⁰ and chemotherapeutics,³¹ all of which are not easily accessible in conventional PEG-grafted lipoplexes. Together, these multifunctional features of the acid-degradable PCL platform bring us closer to an effective siRNA delivery technology that can be utilized for clinical gene therapy. Our future work will focus on increasing the amount of loaded siRNAs in a single delivery particle,³² to achieve better gene knockdown efficiency.

■ ASSOCIATED CONTENT

■ Supporting Information

Experimental and synthetic procedures and full citation for ref 28. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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The authors declare no competing financial interest.

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