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Well-Defined Degradable Cationic Polylactide as Nanocarrier for the Delivery of siRNA to Silence Angiogenesis in Prostate Cancer

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Abstract

Well-defined tertiary amine-functionalized cationic polylactides (CPLAs) are synthesized by thiol-ene click functionalization of an allyl-functionalized polylactide, and utilized here for the delivery of interleukin-8 (IL-8) siRNA via CPLA-IL-8 siRNA nanoplexes. The CPLAs possess remarkable hydrolytic degradability, and their cytotoxicity is relatively low. The CPLA-IL-8 siRNA nanoplexes can be readily taken up by prostate cancer cells, resulting in significant IL-8 gene silencing. It is found that the degradability and cytotoxicity of CPLAs, as well as the transfection efficiency of the CPLA-IL-8 siRNA nanoplexes, positively correlate with the amine mol% of CPLAs.

Keywords

cationic polymer; degradable polymer; prostate cancer; gene delivery; RNAi

1. Introduction

Prostate cancer is the second most common cause of cancer-related mortality in men, which accounts for approximately 32,000 deaths each year.^[1] Nearly 1 in 6 men in the US will be diagnosed with prostate cancer in their lifetime. While the cause of prostate cancer is still being investigated, many early detection techniques and therapeutic strategies for prostate cancer have been developed.^[2] Surgical removal of the prostate, radiation therapy and hormone therapy are the most common clinical treatment options for prostate cancer patients. Although they have been proven to be effective approaches to treat prostate cancer, the quality of life of patients can be seriously affected by surgical or chemical castration. Even after surgical removal of local tumor, more aggressive disease might necessitate chemo- and radi-therapy for metastatic tumors in addition to anti-androgen therapy. Optimal therapy of metastatic prostate cancer remains an elusive goal.

Angiogenesis, a feature of aggressive tumors, is regulated by stimulators and inhibitors of endothelial cell proliferation, migration and invasion.^[3] Angiogenesis facilitates tumor growth by providing oxygenation and nutrition through a series of steps.^[4] We have shown that in prostate cancer, angiogenesis correlates with disease stage and metastasis.^[5] Various growth factors, such as interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), transforming growth factor (TGF)- β and tumor necrosis factor (TNF)- α , can induce angiogenesis.^[6,7] IL-8 promotes prostate tumor growth and modulates the survival and proliferation of tumor cells.^[8] Increased serum levels of IL-8 have been reported in prostate cancer patients in comparison with normal subjects or patients with benign tumors.^[9] IL-8, a member of the CXC chemokine family, is chemotactic and activates leukocytes, with mitogenic and angiogenic properties in a range of human, solid tumor.^[10-22] IL-8 is secreted by various stromal cells (*e.g.* endothelial cells and fibroblasts) and malignant cells (*e.g.* melanoma, prostate and endometrial cancers). IL-8 binds with high specificity to CXCR1 and with less specificity to CXCR2.^[23] The mechanism by which IL-8 promotes tumor growth is believed to be due to an autocrine action of IL-8 in modulating survival and proliferation of tumor cells.^[8] Additionally increased production of IL-8 is believed to be involved in metastasis of cancer cells as well.^[24] It has been documented that expression of IL-8 by CaP cells correlates with angiogenesis, tumorigenicity, and metastasis in mouse models of CaP.^[17,25] These observations are supported by *in vitro* experiments demonstrating that IL-8 increases invasiveness of CaP cells by regulating matrix metalloproteinase synthesis and secretion,^[17, 25] and is associated with progression of the

LNCAp cell line toward androgen-independent growth.^[26] We have reported elevated expression of IL-8 and its receptors, CXCR1 and CXCR2, by CaP cells indicating that they are subject to autocrine/paracrine regulation by IL-8.^[5] Moreover, we found that the most aggressive CaP cells, PC3, manifested the highest expression of IL-8 and its receptors. In addition to acting upon themselves, IL-8 production by CaP cells modulates the activity of other cells within the tumor. The pro-angiogenic activity of IL-8 acts upon endothelial cells of the tumor to induce neovascularization.^[27] Furthermore, the chemotactic activity of IL-8 can recruit neutrophils and macrophages with their tumor-promoting activities.^[28]

Currently, gene therapy in cancer has drawn a great deal of attention because of its revolutionary therapeutic strategy and high specificity. For instance, the RNA-interference (RNAi) mechanism involving the use of small interfering RNA (siRNA) selectively silences specific molecular pathways to overcome critical barriers in cancer treatment. Gene therapy appears to be relatively non-invasive due to its high specificity for the targeted gene. RNAs are extremely susceptible to degradation in vivo and siRNA has limited ability to penetrate the cell membrane efficiently without the assistance of a carrier. Over the past two decades, viral vectors have been demonstrated as promising gene delivery vehicles;^[29] however, some of the bottlenecks, such as immune response, bio-safety concerns and limitations in large-scale production, are still under investigation^[30,31] The dramatic development of nanotechnology has resulted in the resolution of some of these issues by using non-viral vectors,^[32] including lipid-based materials,^[33] cationic polymers,^[34] biodegradable polymers,^[35] inorganic nanoparticles^[36] and gold nanorods.^[37]

Cationic polymers, such as poly(ethylenimine), poly(2-(dimethylamino)ethyl methacrylate), poly-L-lysine and other cationic peptides, have been broadly studied for gene delivery applications.^[31,38] They can allow for the formation of polymer-gene nanoplexes on the order of *ca.* 100 nm with the therapeutic genes through electrostatic interactions. The gene materials can be protected from the nuclease attack by the polymeric scaffolds, thereby resulting in efficient gene delivery into the targeted cells. The cationic polymers with amine groups of pK_a around 5~7 may possess improved transfection results because of the “proton sponge” effect.^[39-42] However, they can be significantly cytotoxic.^[43,44] Especially, their long-term cytotoxicity is a concern, because most of them are non-degradable, and even cationic peptides may not degrade readily under typical biological aqueous conditions.

Polymers with appreciable degradability have also been extensively investigated for gene delivery applications due to their major advantages such as low cytotoxicity, biocompatibility, and protection of therapeutic genes from serum nuclease degradation in blood.^[45] Poly(lactic-co-glycolic acid) (PLGA), an FDA approved biodegradable polymer, has been widely used in gene silencing and therapy.^[46-48] Because PLGA is lipophilic, sophisticated structural design and encapsulation process were required for PLGA-based gene delivery systems to facilitate their water dispersibility; however, their encapsulation efficiency typically was low. Moreover, due to the slow hydrolysis of PLGA, it takes a relatively long time to release the encapsulated payload from the PLGA-based systems, and high levels of target gene expression may not be reached. Furthermore, because of the slow diffusion of acidic degradation products, very acidic gene-damaging environments ($pH < 3$) have been observed in PLGA matrices.^[49,50]

Strategic integration of significant degradability with cationic polymers may create water-soluble biomaterials which not only can complex with genes by electrostatic interaction, but also may possess minimal cytotoxicity under aqueous conditions. A variety of degradable cationic polymers have been synthesized, and their promising applications in gene delivery have been demonstrated.^[51-58] Langer and co-workers have prepared poly(β -amino ester)s by the aminodiacrylate addition reactions, and further screened a large library of such

polymers for DNA complexation and cell transfection.^[51-53] Park, Kim and co-workers have synthesized poly[α -(4-aminobutyl)-L-glycolic acid] via polyesterification, and used the polymer for in vitro and in vivo DNA delivery studies.^[54-56] Poly(4-hydroxy-L-proline ester) has also been prepared via polyesterification, and studied for DNA complexation by multiple groups.^[57,58] In general, ready hydrolytic degradation and insignificant cytotoxicity were observed for these polymers. However, because step polymerization techniques were typically used in their preparation, it remains a challenge to achieve well-defined degradable cationic polymers with tunable cationic densities. The relationship between the densities of cationic moieties and the properties of the polymers making them suitable for gene delivery applications was not reported in these pioneering studies.

With an interest in the novel synthesis and biomedical application of degradable polymers, we recently investigated the synthesis of well-defined degradable cationic polymers based on living polymerization techniques, and their applications as gene delivery scaffolds. In this article, we describe the synthesis of well-defined cationic polylactides (CPLAs) by living ring-opening polymerization (ROP) and thiol-ene click functionalization. In addition, we report on their performance as novel safe nanocarriers for non-invasive cancer gene therapy. In our system, different ratios of tertiary amines were grafted on a degradable polylactide backbone to serve as cationic sources to complex negatively charged siRNA by electrostatic interactions. The siRNAs were well-protected in the resulting nanoplex and the net positive surface charge enabled cell membrane penetration and intracellular trafficking. Our studies show that the transfection efficacy of siRNA is highly correlated with the tertiary amine ratio. To the best of our knowledge, this is the first report on the effects of densities of cationic moieties on degradability and cytotoxicity of degradable cationic polymers, and the transfection efficiency of the derived gene delivery systems.

2. Result and Discussion

2.1. Nanoplex Design for Efficient IL-8 siRNA Delivery

Nanotechnology has become a powerful tool to address many biomedical obstacles due to the unique properties of nanomaterials. To overcome the challenge that naked siRNA is susceptible to degradation and cannot penetrate the cell membrane easily and efficiently, we designed a nanotherapeutic system based on CPLA. The concept of gene delivery using CPLA is illustrated in Scheme 1. The negatively charged IL-8 siRNA is complexed with CPLA by electrostatic interaction. A portion of the positive charges on CPLA is neutralized by IL-8 siRNA, and the nanoplexes formed not only provide protection of siRNA but also possess a net positive charge that can enhance cellular internalization via endocytosis. When the nanoplex is being transported in the cytoplasm, the tertiary amine groups of CPLA appear on the nanoplex surface to endow a proper extent of the intracellular “proton-sponge” effect, whereby extra protons are transported and absorbed into the endosome, inducing osmotic swelling and rupture.^[30,42,59-61] The nanoplexes eventually escape from the endosome and the IL-8 siRNA is released upon the degradation of CPLA. In order to achieve efficient delivery of siRNA via such a design, several factors must be taken into account:

- i. Degradability of the nanocarrier. It is well known that an ideal strategy for siRNA delivery involves the degradation of nanocarrier after a successful delivery of the payload.^[30] At the same time, the degradation rate of the material affects the efficiency of delivery. If the material degrades too fast, siRNA will not be effectively protected. On the other hand, if the degradation does not occur after the endosomal escape, RNAi function will be impeded;

- ii. Protection of siRNA. The siRNA should be able to attach to the polymeric scaffold to prevent its degradation by the extracellular medium, intracellular enzymatic degradation and the acidic endosomal environment;
- iii. Positive surface charge. Cellular uptake can be enhanced by a net positive surface charge on the nanoplex;^[62-64]
- iv. “Proton-sponge” effect. The presence of protonable amine (e.g. tertiary amine) in the nanocarrier can act as a weak base to absorb protons generated by ATPase, which facilitates endosomal escape and improves the transfection efficiency;^[30, 42, 59-61]
- v. Low-cytotoxicity. For safe delivery, the introduction of nanocarriers to the biological milieu should not induce considerable toxicity in vitro and in vivo.

2.2. Synthesis of CPLAs

As compared with step polymerization, living ROP can yield well-defined polymers with degradable in-chain functionalities.^[65] Recent developments of click chemistries have provided powerful tools for polymer synthesis and functionalization.^[66] Therefore, a synthetic route via tandem living ROP and click chemistries was designed to achieve well-defined CPLAs (Scheme 2). Further, the organocatalyzed ROP process and thiol-ene click reaction were selected, because they can be conducted under mild conditions, without using toxic metal-based catalyst.^[67,68] Correspondingly, a series of CPLAs with different densities of cationic groups were prepared through the ROP synthesis of an alkene-functionalized PLA, followed by its reaction with a thiol-functionalized cationic agent. The allyl functionality was chosen as the alkene group of the functional PLA due to its high reactivity in thiol-ene reaction.^[69] To induce the “proton sponge” effect with reduced toxicity relative to primary and secondary amines, a tertiary amine group was chosen as the cationic group for the CPLAs; 2-(diethylamino)ethanethiol hydrochloride (DEAET) was further selected as the thiol-containing functionalization agent.^[70,71]

The ROP of allyl-functionalized lactide (LA) monomer **1** with L-LA was conducted using benzyl alcohol (BnOH) as the initiator and 4-dimethylaminopyridine (DMAP) as the organocatalyst ($[1]_0:[LA]_0:[BnOH]_0:[DMAP]_0 = 50:50:1:4$; 35 °C, 168 h, in dichloromethane (DCM); ~90% overall conversion of comonomers).^[69] The well-defined chemical structure of the resulting allyl-functionalized PLA **2** was verified by ¹H NMR (Figure 1a) and GPC (Figure 2) characterizations. Based on the resonance intensities of the allyl CH protons (CH=CH₂) from the monomer unit of **1** at 5.77-5.79 ppm and the CH protons from both co-monomers and the allyl CH₂ protons (CH=CH₂) from monomer unit of **1** at 5.14-5.23 ppm, a molar fraction of 50% for the monomer unit of **1** in the polymer **2** was deduced, which agreed precisely with the 50 mol% feed ratio of **1** for copolymerization. The number-average degree of polymerization (DP_n) of **2** was determined by comparing the resonance intensities of the CH protons from the comonomer units and the allyl CH₂ protons (CH=CH₂) from monomer unit of **1** at 5.14-5.23 ppm with the resonance intensities of the terminal C₆H₅ protons from benzyl alcohol at 7.35 ppm. The experimental DP_n of 80 was relatively close to the theoretical DP_n of ~90 for **2**. From the experimental DP_n and the composition of **2**, its number-average molecular weight (*M_n*) of 12.7 kDa was further obtained. As determined by the GPC analysis, **2** possessed a *M_n* of 12.0 kDa and a low polydispersity (PDI) of 1.16 relative to linear polystyrene.

Sequentially, the UV-induced thiol-ene reactions of the allyl-functionalized PLA **2** with DEAET were performed in CDCl₃ under UV irradiation for 30 min at room temperature, using 2,2'-dimethoxy-2-phenylacetophenone (DMPA) as the photoinitiator.^[69,71] By altering the [ene]₀:[SH]₀:[DMPA]₀ feed ratio, a series of well-defined CPLAs with different

amounts of tertiary amine groups were successfully synthesized (Table 1). With 9, 18, 30, and 50 mol% of amine functionalities relative to polymer backbone repeat units, these polymers were denoted as CPLA-9, CPLA-18, CPLA-30 and CPLA-50. ^1H NMR analysis was used to determine their composition. For instance, the ^1H NMR spectrum of CPLA-30 was shown in Figure 1b, and its mole fractions of **1** and the tertiary amine-based unit were 20% and 30% respectively, based on a comparison of the resonance intensities of allyl CH protons ($\text{CH}=\text{CH}_2$) from the monomer unit of **1** (at 5.78 ppm) and the methyl protons of the tertiary amine-based unit (at 1.21-1.30 ppm). All CPLA samples were also characterized by GPC (Figure 2). The peak elution volumes (at 17.5-17.6 mL) and M_n^{GPC} values (10.5-12.1 kDa) of CPLAs were close to that of allyl-functionalized PLA **2** (17.7 mL, 12.0 kDa). It can be inferred that the transformation from the allyl functionalities to the tertiary amine groups did not result in significant change on the hydrodynamic volumes of these polymers in the GPC eluent (N,N-dimethylmethanamide (DMF) with 0.1 M LiBr at 57 °C). CPLAs showed only a slightly higher PDI^{GPC} value (1.20-1.23) than the precursor polymer **2** ($PDI^{\text{GPC}} = 1.16$). The result suggested that the occurrence of side reactions was well suppressed under the reaction conditions of thiol-ene functionalization.

2.3 Degradation of CPLAs

Degradability of non-viral vectors under biological aqueous conditions is critically important for their successful application as delivery scaffolds for gene therapies. After the nanoplex is internalized inside the cells, the degraded polymeric scaffolds can more readily unload genes into the cytoplasm.^[30,34] The degradability of water-insoluble polymers was typically assessed by the mass loss, but this method was unable to monitor the backbone cleavage events of the polymers during the hydrolytic degradation process.^[72,73] In this study, gel permeation chromatography (GPC) was selected as the analytic tool to study the hydrolytic degradability of all water-soluble CPLA samples, including CPLA-18, CPLA-30 and CPLA-50. The backbone cleavage events of these CPLAs during the degradation process can be effectively probed through the increases of elution volumes of the polymer species and the changes of peak elution volumes in the GPC curves of their degradation residues relative to the original polymers.^[74]

The degradation study was conducted by the incubation of 1.0 mg/mL of CPLAs at 37 and 25 °C in de-ionized water in sealed vials, followed by withdrawing aliquots at 1, 5, 9, 13 h and 1 week for the GPC analysis. From the GPC curves shown in Figure 3, it was observed that CPLAs were readily degradable in the aqueous solution, and their degradation rates significantly increased with the increases of their amine mol% and hydrolysis temperature. CPLA-18 showed a significant degradation after 13 h at 37 °C, with the formation of a minor elution peak at 18.7 mL. The degradation of CPLA-30 was faster than CPLA-18, as indicated by its major elution peak at 19.7 mL after 13 h at 37 °C. Among the CPLA samples, CPLA-50 showed the fastest degradation under the same conditions. At 37 °C in deionized water, the degradation of CPLA-50 resulted in a minor elution peak at 20.0 mL after 1 h, a major elution peak at 20.9 mL after 5 h, and complete disappearance of the initial elution peak of 17.7 mL after 9 h. The degradation of CPLA-50 was also relatively fast at 25 °C, and its initial elution peak of 17.7 mL essentially disappeared after 13 h. For each of the CPLA samples with long incubation times, oligomeric residuals with elution volumes around 20.8 mL were also observed, besides small molecule degradation products which were eluted with solvent at ~22 mL. Analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), these oligomers had molecular weights around only 1 kDa (see Figure S1).

The fast degradation of CPLAs indicated that the main chain cleavages are not only caused by the hydrolysable ester bonds, but also affected by the nucleophilic attack of the tertiary

amines.^[54,58] The nucleophilic attack may occur either intra- or intermolecularly, resulting in ready degradation with rates increasing with the amine mol% of CPLAs. According to the dependence of the degradation rate on the amine mol% of CPLA, their degradation properties may be tailored by controlling their amine mol%. This feature is desirable for the design of a gene delivery system, because the desired degradation time frame of polymeric scaffolds can be obtained by selecting the CPLA with an optimal composition in terms of the practical needs of biological systems.

The hydrolysis of CPLAs did not result in a highly acidic aqueous solution. The pH values of the solutions of CPLAs in deionized water were close to 7.0 at the beginning, decreased gradually during the hydrolysis process, and could reach ~5.5 with the occurrence of high extent of hydrolysis. As compared with the low pH values (< 3) observed in hydrolyzed PLGA matrices,^[49] the acidity resulting from CPLA aqueous degradation is relatively mild and unlikely would cause gene damage when CPLA is used as a delivery scaffold.

2.4 Cytotoxicity of CPLAs

Evaluation of the cytotoxicity of biodegradable nanocarriers is crucial for their ultimate use for biomedical and clinical applications. The MTS cell viability assay (Promega, Madison, WI) was used to determine the effect of CPLAs on cell viability in vitro experiments. Dose-dependent studies on CPLAs were carried out with the prostate cancer cell line, PC3. Untreated control PC3 cells served as the reference (i.e. 100% cell viability). Results from the MTS assay (Figure 4) showed that the treatment of PC3 cells with a high dosage of CPLA-18 and CPLA-30 over a time period of 48 h did not result in any cytotoxicity. It is worth mentioning that the dosage of 300 $\mu\text{g/mL}$ is very high as compared with the maximum safe dosages of only 10 $\mu\text{g/mL}$ for poly(ethylenimine) reported in the literature.^[48,75] However, some toxicity was observed (70% viability) with CPLA-50 at this dosage. It can be due to the fact that the higher positive charge ratio enhanced the cellular uptake thus increasing the toxic effect on the cells. It is noted that the cell viability was ~100% at 100 $\mu\text{g/mL}$ and greater than 90% even at 200 $\mu\text{g/mL}$, indicating the CPLA-50 is relatively safe to use for typical in vitro studies.

2.5 Nanoplex Formation, Cellular Uptake and Transfection Efficiency

Using the weight ratio of CPLA/siRNA of 20:1, CPLA-IL-8 siRNA nanoplexes were prepared by electrostatic interaction between CPLA and negatively charged IL-8 siRNA (see the Experimental section). With a portion of the positive charge on the CPLA backbones neutralized, the nanoplex can protect IL-8 siRNA within the polymeric matrix to prevent siRNA degradation. All water-soluble CPLA samples, including CPLA-18, CPLA-30 and CPLA-50, were used for nanoplex preparation. However, because of a limited availability of IL-8 siRNA, only CPLA-50-IL-8 siRNA, as the representative nanoplex sample, was prepared in a sufficient amount for comprehensive characterization to reveal the general structural features of these nanoplexes. As measured by dynamic light scattering (DLS), the CPLA-50-IL-8 siRNA nanoplex had a volume-average hydrodynamic diameter ($D_{h,v}$) of 63.3 ± 2.2 nm, with a relatively low PDI of 0.21 (Figure 5a). The nanoplex also possessed a zeta-potential of $+12.7 \pm 1.4$ mV, due to the presence of the tertiary amine groups from CPLA on the nanoplex surface. Such a net positive surface charge can prevent the nanoplex from aggregating. The nanostructure of CPLA-50-IL-8 siRNA was further visualized by transmission electron microscopy (TEM) using RuO_4 as the staining agent (Figure 5b). A round surface morphology was observed for CPLA-50-IL-8 siRNA, and the average surface diameter of 48.1 ± 10.2 nm was comparable to the $D_{h,v}$ value obtained by DLS.

Therapeutic applications of IL-8 siRNA gene therapy for prostate cancer treatment have been reported.^[76-78] To elucidate the effect of CPLA-IL-8 siRNA nanoplexes on gene

silencing of IL-8 for the purpose of gene therapy for cancer, different CPLAs as carriers were compared with a commercially available transfection reagent, Mirus TransIT (Mirus, Madison, WI), in terms of the efficiency of IL-8 gene suppression. PC3 cells were incubated in culture medium containing 10% fetal bovine serum (FBS) with the CPLA-IL-8 siRNA nanoplexes and appropriate controls (untransfected, Mirus TransIT with IL-8 siRNA, Mirus TransIT with scrambled siRNA, CPLAs only, and naked IL-8 siRNA) for 48 h and the efficiency of gene silencing was determined by measuring the percentage inhibition of the expression of IL-8 using real-time quantitative polymerase chain reaction (Q-PCR). Cells treated with naked IL-8, free CPLAs, and Mirus TransIT with scrambled siRNA only showed negligible suppression (< 10% knockdown) of the IL-8 gene, while cells treated with CPLA-18-IL-8 siRNA, CPLA-30-IL-8 siRNA and CPLA-50-IL-8 siRNA exhibited considerable suppression of IL-8 gene expression by PC3 (Figure 6). Moreover, CPLA-50-IL-8 siRNA (52% knockdown) had a higher transfection efficiency than CPLA-30-IL-8 siRNA (25% knockdown) and CPLA-18-IL-8 siRNA (17% knockdown). The enhanced gene delivery and knockdown might be partly due to the comprehensive effects of the higher charged ratio of CPLA-50. Upon internalization of CPLA-50-IL-8 siRNA, the nanoplexes were confined in the endosomal compartments. The unpacking and the endosomal escape of IL-8 siRNA are required to initiate the RNAi process. In our design, CPLA-50 possessed a higher tertiary amine content which can facilitate the endosomal escape by the proton sponge effect.^[79,80] In addition, as shown in Figure 3, CPLA-50 was completely degraded into oligomers within 9 h, indicating that most of the IL-8 siRNA was readily unpacked from the carrier into the cytoplasm which led to an enhanced gene silencing efficiency. It should be noted that the gene silencing efficiency of the IL-8 siRNA delivery via CPLA-50 was even higher than that via the commercial transfection agent Mirus TransIT (39% knockdown).

To visualize the delivery of IL-8 siRNA via nanoplex into cells, CPLA-50 was complexed with Cy5-labeled IL-8 siRNA. CPLA-50-IL-8 siRNA^{Cy5} was prepared and its internalization into the PC3 cells was monitored using confocal microscopy (Figure 7). After the incubation of the PC3 cells with CPLA-50-IL-8 siRNA^{Cy5} for 4 h, strong fluorescence (red) signals from the IL-8 siRNA^{Cy5} were observed (Figure 7). The overlaid fluorescence and bright field images further revealed that most of CPLA-50-IL-8^{Cy5} was uptaken into the cytoplasm via endocytosis. Free CPLA-50 and naked IL-8 siRNA^{Cy5} were used as controls in the cell internalization study. The trials using free CPLA-50 showed no fluorescence signals. Under the same conditions, only minimal uptake of naked IL-8^{Cy5} was observed, presumably because the uptake of negatively charged IL-8 siRNA^{Cy5} was impeded by the cell membrane. The internalized unprotected IL-8 siRNA^{Cy5} may also degrade relatively quickly in the endosomal compartment before reaching the target.

3. Conclusion

Tandem living ROP and click functionalization strategy were utilized for the preparation of degradable cationic polymers. Well-defined CPLAs were successfully obtained through thiol-ene reaction of a tertiary amine-based thiol with an allyl-functionalized polylactide. Their amine amounts were effectively controlled by adjusting the feed ratio of reagents. The significant hydrolytic degradability and the relatively low cytotoxicity of CPLAs were verified. Both the degradability and the cytotoxicity of CPLAs increased with an increase of the amine mol% of the polymers.

A well-defined biodegradable nanopatform using CPLAs as scaffolds for gene delivery was developed. Our design satisfied the essential requirements of safe and efficient nanocarriers for gene delivery. The formation of nanoplexes of CPLA with IL-8 siRNA and ready uptake of the nanoplex by PC3 prostate cancer cells were observed. The efficacy of gene

suppression was positively correlated with the density of cationic groups in the nanocarrier. An increase of IL-8 siRNA intracellular trafficking and enhanced knockdown of IL-8 gene expression in the PC3 cells were successfully demonstrated. The obtained results revealed that our formulation could be a potential candidate for developing a unique, safe and biodegradable nanotherapeutic tool for local and metastatic prostate cancer cells, by suppressing the gene for IL-8 which is required for their growth and metastasis. Furthermore, for in vivo or eventual clinical use, our design may be modified through PEGylation to achieve long circulation times of the nanoplexes.^[81-83] Additional ligands, such as monoclonal antibodies or peptides, can be added to the nanoplexes to target specific cells and tissues of interest. The investigation to synthesize biodegradable nanomaterials with a cargo loading (e.g. ligand, drug and gene) capability for both in vitro and in vivo targeting is ongoing in our laboratories.

4. Experimental Section

Measurements

All ^1H NMR spectra were recorded at 500 MHz on solutions in CDCl_3 on a Varian INOVA-500 spectrometer maintained at 25 °C, with tetramethylsilane (TMS) as an internal reference standard. Gel permeation chromatography (GPC) data were obtained from a Viscotek GPC system equipped with a VE-3580 refractive index (RI) detector, a VE 1122 pump, and two mixed-bed organic columns (PAS-103M and PAS-105M). DMF (HPLC) containing 0.1 M LiBr was used as the mobile phase with a flow rate of 0.5 mL/min at 57 °C. The GPC instrument was calibrated using narrowly-dispersed linear polystyrene standards purchased from Varian.

The average hydrodynamic diameters (D_h) and zeta potential of nanoplexes were measured by dynamic light scattering (DLS) on a nano-ZS90 (Malvern, Inc.) in water at 25 °C. All experiments were conducted using a 4 mW 633 nm HeNe laser as the light source at a fixed measuring angle of 90° to the incident laser beam. The correlation decay functions were analyzed by cumulants method coupled with Mie theory to obtain volume and number distribution. All the samples were passed through a 0.45 μm filter and treated with ultrasonification for 2 min before the measurement.

TEM images were obtained using a JEOL 100CX microscope. TEM samples were prepared by dip coating a 300 mesh carbon-coated copper grid with a dilute sample solution, followed by staining using ruthenium tetroxide. MALDI-TOF was measured on a Bruker Biflex IV (Billerica, MA) MALDI mass spectrometer equipped with a nitrogen laser ($\lambda = 337 \text{ nm}$), using trans-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene] malononitrile (DCTB, Aldrich; 99%) as matrix and sodium trifluoroacetate (NaTFA, Aldrich; ~98%) as a cationizing agent.

Cell imaging was carried out using a Leica TCS-SP2/AOBS confocal microscope, equipped with excitation laser lines of 405, 442, 458, 476, 488, 496, 543, 633 nm and capable of spectral detection in the range of 400-720 nm. All of the confocal images were taken under the same conditions (the parameters of photodetector gain, pinhole size and exposure time were kept constant).

UV irradiation was conducted using a UVGL-58 handheld UV lamp (UVP ultraviolet lamps; 6 Watt, 0.12 Amps) with λ_{max} of 365 nm. Dialysis was carried out against acetone using molecularporous membrane tubing (Spectra/Por Dialysis Membrane, Spectrum Laboratories Inc.) with an approximate molecular weight cut off (MWCO) at 3500 Daltons.

Materials

4-Dimethylaminopyridine (DMAP; 99+%) and L-lactide (L-LA) (98%) were purchased from Sigma-Aldrich. 2,2'-Dimethoxy-2-phenylacetophenone (DMPA; 98%) was purchased from Acros Organics. Dichloromethane (DCM; HPLC), acetone (HPLC), ethyl acetate (HPLC), DMF (HPLC) and BnOH (HPLC) were purchased from Fisher Chemical. 2-(Diethylamino)ethanethiol hydrochloride (DEAET, 98+%) was purchased from Amfinecom Inc. DCM, DMF and ethyl acetate were dried by distillation over CaH₂. LA was recrystallized from dry ethyl acetate 4 times prior to be used. Allyl-functionalized LA monomer **1** was prepared through the method we reported previously.^[69] All chemicals were used without further purification unless stated otherwise.

Synthesis of Poly(1_{0.5}-co-LA_{0.5})₈₀, (**2**)

In a 10 mL reaction flask with a magnetic stirring bar under nitrogen atmosphere, **1** (674 mg; 3.96 mmol), LA (571 mg; 3.96 mmol) and DCM (5.88 mL) were added. The solution was heated to 35 °C using an oil bath, and then benzyl alcohol (8.6 mg, 0.079 mmol; in 0.5 mL DCM) and DMAP (38.7mg, 0.32 mmol; in 0.5mL DCM) were added to initiate the polymerization. After heating at 35 °C for 1 week, the reaction mixture was precipitated in cold methanol (50 mL). The reaction was stopped at comonomer conversion of ~90%. The conversion was determined by ¹H NMR spectroscopy of an aliquot of polymerization solution, based on the resonance intensities of the CH₃ protons of remaining comonomers at 1.67-1.73 ppm relative to the CH₃ protons of the resulting polymer at 1.49-1.61 ppm. The precipitate was collected by filtration and dried in a vacuum to give **2** as a white solid in 70% isolated yield. ¹H NMR (500 MHz, CDCl₃, ppm): δ 1.49-1.61 (br m, CH₃ of units from LA and **1**), 2.66-2.73 (br m, CH₂CH=CH₂ of units from **1**), 5.14-5.23 (br m, CHCH₃ of units from LA, CHCH₃, CHCH₂CH=CH₂, and CH₂CH=CH₂ of units from **1**), 5.77-5.79 (m, CH₂CH=CH₂ of units from **1**), 7.33-7.39 (m, Ar-H from BnOH). *M_n*^{NMR} = 12.7 kDa, *M_n*^{GPC} = 12.0 kDa, *PD*^{GPC} = 1.16. Mole fraction of **1** were 50% based upon the ¹H NMR resonance intensities of 1H from units of **1** at 5.77-5.79 ppm relative to 4H from units of **1** and 2H from units of LA at 5.14-5.23 ppm.

Synthesis of CPLAs

The experimental procedure for the preparation of CPLA-30 is given, and all other CPLA samples were prepared using the same method. In a 50 mL flask, **2** (100 mg), DEAET (48 mg) and photoinitiator DMPA (24.3 mg) were dissolved in CDCl₃ (10 mL) with the molar ratio of [allyl of **2**]₀: [SH of DETA]₀: [DMPA]₀ = 1:0.9:0.3. To remove the oxygen in the reactant solution, a freeze-pump-thaw procedure was conducted for 3 cycles. Then, thiol-ene reaction was induced by UV irradiation (λ_{max} = 365 nm) for 30 min. The reactant solution was dialyzed against acetone for 10 days and then dried in vacuo to give CPLA-30 with 90% yield. ¹H NMR (500 MHz, CDCl₃, ppm): δ 1.21-1.26 (br m, (CH₃CH₂)₂NH⁺Cl⁻ from amine-functionalized units), 1.55-1.59 (br m, CH₃ of units from LA and **1**), 1.76-1.77 (br m, CH₂CH₂CH₂SCH₂ from amine-functionalized units), 2.00-2.08 (br m, CH₂CH₂CH₂SCH₂ from amine-functionalized units), 2.49-3.20 (br m, CH₂CH=CH₂ of units from **1** and CH₂CH₂CH₂SCH₂, SCH₂CH₂NH⁺Cl⁻(CH₂CH₃)₂ from amine-functionalized units), 5.14-5.20 (br m, CHCH₃ of units from LA, CHCH₃, CHCH₂CH=CH₂, and CH₂CH=CH₂ of units from **1**), 5.77-5.79 (br m, CH₂CH=CH₂ of units from **1**), 7.30-7.37 (m, Ar-H from BnOH).

Preparation of CPLA-IL-8 siRNA Nanoplexes

IL-8 siRNA (5' GGAUUUCCUAGAUAUUGC dtdt) was obtained from Thermo Scientific Dharmacon. A 27μl aliquot of CPLA stock solution (CPLA-18, CPLA-30 and CPLA-50, 1.0 mg/mL) was mixed with 10 μl of IL-8 siRNA (10μM) (CPLA/siRNA weight

ratio = 20:1) with a gentle vortex. The mixture was left undisturbed for 30 min for the formation of the CPLA-IL-8 nanoplex.

Cell Viability Study

The cell viability assay measures the reduction of a tetrazolium component 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, or MTS into a formazan product by the mitochondria of viable cells. Cells in a 96-well plate (~5,000 PC3 cells/well) were incubated with different concentrations of CPLAs for 48 h at 37 °C in a humidified air plus 5% CO₂ atmosphere. Untreated cells served as the control. The absorbance of formazan (produced by the cleavage of MTS by dehydrogenases in living cells) is directly proportional to the number of live cells. After the incubation, MTS reagent was added to each well. After 1.5 h, the absorbance of sample at 490 nm was recorded using a multiwell plate reader (Opsys MR™, Dynex).

Transfection

Transgene expression was monitored at 48 h post-treatment. At 24 h before siRNA transfection, ~20,000 PC3 cells were seeded onto 6-well plates in OPTI-MEM medium containing 4% FBS with no antibiotics to give 30 to 50% confluence at the time of transfection. The siRNA was reconstituted in DNase-RNase free water to a final concentration of 10 μM and mixed with 27 μl of CPLA solution as described above. The final concentration of siRNA for in vitro transfection of cancer cells was 100 nM. The commercially available siRNA delivery agent, Mirus TransIT-TKO was used as a positive control in the experiments.

RNA extraction and real-time quantitative polymerase chain reaction (Q-PCR)

Cytoplasmic RNA was extracted by an acid guanidinium-thiocyanate-phenol-chloroform method,^[84] using the TRIzol® reagent (Invitrogen, Carlsbad, CA) and the final RNA pellet was dried and resuspended in diethyl pyrocarbonate (DEPC)-treated water and the concentration of RNA was determined using a spectrophotometer at 260 nm. DNA contamination in the RNA preparation was removed by treating the RNA preparation with DNase (1 IU/μg of RNA, Promega, Madison, WI) for 30 min at 37°C, followed by proteinase K digestion at 37°C for 15 min and subsequent extraction with phenol/chloroform and NH₄OAc/EtOH precipitation. The precipitated RNA was stored at -70 °C until used. Relative expression of IL-8 mRNA was assessed using the SYBR green master mix from Stratagene (La Jolla, CA) to perform Q-PCR using the Stratagene MX3005B (La Jolla, CA) instrument that detects and plots the increase in fluorescence versus PCR cycle number to produce a continuous measure of PCR amplification. To provide precise quantification of initial target in each PCR reaction, the amplification plot is examined at a point during the early log phase of product accumulation. This is accomplished by assigning a fluorescence threshold above background and determining the time point at which each sample's amplification plot reaches the threshold (defined as the threshold cycle number or C_T). Differences in threshold cycle number is used to quantify the relative amount of PCR target contained within each tube.^[85] Relative mRNA species expression is quantitated and expressed as transcript accumulation index (TAI = 2 – delta delta C_T), calculated using the comparative C_T method.^[86] All data were controlled for quantity of RNA input by performing measurements on an endogenous reference gene, β-actin. In addition, results on RNA from treated samples were normalized to results obtained on RNA from the control, untreated sample.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Cancer Facts & Figures 2010. American Cancer Society;
2. Law WC, Yong KT, Baev A, Prasad PN. ACS Nano. 2011; 5:4858. [PubMed: 21510685]
3. Liotta LA, Steeg PS, Stetlerstevenson WG. Cell. 1991; 64:327. [PubMed: 1703045]
4. Izawa JI, Dinney CPN. Can Med Assoc J. 2001; 164:662. [PubMed: 11258215]
5. Aalinkel R, Nair MP, Sufrin G, Mahajan SD, Chadha KC, Chawda RP, Schwartz SA. Cancer Res. 2004; 64:5311. [PubMed: 15289337]
6. Folkman J, Klagsbrun M. Science. 1987; 235:442. [PubMed: 2432664]
7. Folkman J, Natl J. Cancer Inst. 1990; 82:4.
8. Wang JM, Deng XY, Gong WH, Su SB, Immunol J. Methods. 1998; 220:1.
9. McCarron SL, Edwards S, Evans PR, Gibbs R, Dearnaley DP, Dowe A, Southgate C, Easton DF, Eeles RA, Howell WM. Cancer Res. 2002; 62:3369. [PubMed: 12067976]
10. Chen Z, Malhotra PS, Thomas GR, Ondrey PG, Duffey DC, Smith CW, Enamorado N, Yeh NT, Kroog GS, Rudy S, McCullagh L, Mousa S, Quezado M, Herscher LL, Van Waes C. Clin Cancer Res. 1999; 5:1369. [PubMed: 10389921]
11. Davidson B, Goldberg I, Kopolovic J, Gotlieb WH, Givant-Horwitz V, Nesland JM, Berner A, Ben-Baruch G, Bryne M, Reich R. Clin Exp Metastasis. 2001; 18:501. [PubMed: 11592307]
12. Fujimoto J, Aoki I, Khatun S, Toyoki H, Tamaya T. Ann Oncol. 2002; 13:430. [PubMed: 11996475]
13. Fujimoto J, Sakaguchi H, Aoki I, Tamaya T. Cancer Res. 2000; 60:2632. [PubMed: 10825134]
14. Galffy G, Mohammed KA, Dowling PA, Nasreen N, Ward MJ, Antony VB. Cancer Res. 1999; 59:367. [PubMed: 9927048]
15. Green AR, Green VL, White MC, Speirs V. Int J Cancer. 1997; 72:937. [PubMed: 9378554]
16. Haraguchi M, Komuta K, Akashi A, Matsuzaki S, Furui J, Kanematsu T. Oncol Rep. 2002; 9:159. [PubMed: 11748475]
17. Inoue K, Slaton JW, Eve BY, Kim SJ, Perrotte P, Balbay MD, Yano S, Bar-Eli M, Radinsky R, Pettaway CA, Dinney CPN. Clin Cancer Res. 2000; 6:2104. [PubMed: 10815938]
18. Kitadai Y, Haruma K, Sumii K, Yamamoto S, Ue T, Yokozaki H, Yasui W, Ohmoto Y, Kajiyama G, Fidler IJ, Tahara E. Am J Pathol. 1998; 152:93. [PubMed: 9422527]
19. Masood R, Cai J, Tulpule A, Zheng T, Hamilton A, Sharma S, Espina BM, Smith DL, Gill PS. Clin Cancer Res. 2001; 7:2693. [PubMed: 11555581]
20. Ren Y, Poon RTP, Tsui HT, Chen WH, Li Z, Lau CL, Yu WC, Fan ST. Clin Cancer Res. 2003; 9:5996. [PubMed: 14676125]
21. Slaton JW, Inoue K, Perrotte P, El-Naggar AK, Swanson DA, Fidler IJ, Dinney CPN. Am J Pathol. 2001; 158:735. [PubMed: 11159211]
22. Yuan A, Yu CJ, Luh KT, Kuo SH, Lee YC, Yang PC. J Clin Oncol. 2002; 20:900. [PubMed: 11844810]
23. Rossi D, Zlotnik A. Annu Rev Immunol. 2000; 18:217. [PubMed: 10837058]
24. De Larco JE, Wuertz BRK, Yee D, Rickert BL, Furcht LT. Proc Natl Acad Sci USA. 2003; 100:13988. [PubMed: 14623984]
25. Kim SJ, Uehara H, Karashima T, Mccarty M, Shih N, Fidler IJ. Neoplasia. 2001; 3:33. [PubMed: 11326314]
26. Reiland J, Furcht LT, McCarthy JB. Prostate. 1999; 41:78. [PubMed: 10477904]
27. Mizukami Y, Jo WS, Duerr EM, Gala M, Li JN, Zhang XB, Zimmer MA, Iliopoulos O, Zukerberg LR, Kohgo Y, Lynch MP, Rueda BR, Chung DC. Nat Med. 2005; 11:992. [PubMed: 16127434]
28. De Larco JE, Wuertz BRK, Furcht LT. Clin Cancer Res. 2004; 10:4895. [PubMed: 15297389]

29. During MJ. *Adv Drug Deliver Rev.* 1997; 27:83.
30. Pack DW, Hoffman AS, Pun S, Stayton PS. *Nat Rev Drug Discov.* 2005; 4:581. [PubMed: 16052241]
31. Mintzer MA, Simanek EE. *Chem Rev.* 2009; 109:259. [PubMed: 19053809]
32. Musacchio T, Navro G, Torchilin VP. *J Drug Del Sci Tech.* 2012; 22:5.
33. Li WJ, Szoka FC. *Pharm Res.* 2007; 24:438. [PubMed: 17252188]
34. Jeong JH, Kim SW, Park TG. *Prog Polym Sci.* 2007; 32:1239.
35. Luten J, van Nostruin CF, De Smedt SC, Hennink WE. *J Control Release.* 2008; 126:97. [PubMed: 18201788]
36. Trewyn BG, Slowing II, Giri S, Chen HT, Lin VSY. *Acc Chem Res.* 2007; 40:846. [PubMed: 17645305]
37. Bonoiu AC, Mahajan SD, Ding H, Roy I, Yong KT, Kumar R, Hu R, Bergey EJ, Schwartz SA, Prasad PN. *Proc Natl Acad Sci USA.* 2009; 106:5546. [PubMed: 19307583]
38. Gabrielson NP, Lu H, Yin L, Li D, Wang F, Cheng J. *Angew Chem Int Ed.* 2012; 51:1143.
39. Yu HJ, Zou YL, Wang YG, Huang XN, Huang G, Sumer BD, Boothman DA, Gao JM. *Acs Nano.* 2011; 5:9246. [PubMed: 22011045]
40. Patil ML, Zhang M, Minko T. *ACS Nano.* 2011; 5:1877. [PubMed: 21322531]
41. Creusat G, Rinaldi AS, Weiss E, Elbaghdadi R, Remy JS, Mulherkar R, Zuber G. *Bioconjugate Chem.* 2010; 21:994.
42. Boussif O, Lezoualch F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP. *Proc Natl Acad Sci USA.* 1995; 92:7297. [PubMed: 7638184]
43. Fang H, Zhang K, Shen G, Wooley KL, Taylor JSA. *Mol Pharm.* 2009; 6:615. [PubMed: 19231840]
44. Wu J, Lizarzaburu ME, Kurth MJ, Liu L, Wege H, Zern MA, Nantz MH. *Bioconjugate Chem.* 2001; 12:251.
45. Nguyen DN, Green JJ, Chan JM, Longer R, Anderson DG. *Adv Mater.* 2009; 21:847.
46. Cun DM, Foged C, Yang MS, Frokjaer S, Nielsen HM. *Int J Pharm.* 2010; 390:70. [PubMed: 19836438]
47. Woodrow KA, Cu Y, Booth CJ, Saucier-Sawyer JK, Wood MJ, Saltzman WM. *Nat Mater.* 2009; 8:526. [PubMed: 19404239]
48. Nguyen J, Steele TWJ, Merkel O, Reul R, Kissel T. *J Control Release.* 2008; 132:243. [PubMed: 18619502]
49. Fu K, Pack DW, Klibanov AM, Langer R. *Pharm Res.* 2000; 17:100. [PubMed: 10714616]
50. Walter E, Moelling K, Pavlovic J, Merkle HP. *J Control Release.* 1999; 61:361. [PubMed: 10477808]
51. Lynn DM, Langer R. *J Am Chem Soc.* 2000; 122:10761.
52. Anderson DG, Lynn DM, Langer R. *Angew Chem Int Ed.* 2003; 42:3153.
53. Zugates GT, Anderson DG, Little SR, Lawhorn IEB, Langer R. *J Am Chem Soc.* 2006; 128:12726. [PubMed: 17002366]
54. Lim YB, Kim CH, Kim K, Kim SW, Park JS. *J Am Chem Soc.* 2000; 122:6524.
55. Lim YB, Han SO, Kong HU, Lee Y, Park JS. *Pharm Res.* 2000; 17:811. [PubMed: 10990199]
56. Koh JJ, Ko KS, Lee M, Han S, Park JS, Kim SW. *Gene Ther.* 2000; 7:2099. [PubMed: 11223991]
57. Putnam D, Langer R. *Macromolecules.* 1999; 32:3658.
58. Lim YB, Choi YH, Park JS. *J Am Chem Soc.* 1999; 121:5633.
59. Ong ZY, Fukushima K, Coady DJ, Yang YY, Ee PLR, Hedrick JL. *J Control Release.* 2011; 152:120. [PubMed: 21277917]
60. Tamura A, Oishi M, Nagasaki Y. *J Control Release.* 2010; 146:378. [PubMed: 20621664]
61. Yezhelyev MV, Qi LF, O'Regan RM, Nie S, Gao XH. *J Am Chem Soc.* 2008; 130:9006. [PubMed: 18570415]
62. Peng SF, Su CJ, Wei MC, Chen CY, Liao ZX, Lee PW, Chen HL, Sung HW. *Biomaterials.* 2010; 31:5660. [PubMed: 20399497]

63. Perumal OP, Inapagolla R, Kannan S, Kannan RM. *Biomaterials*. 2008; 29:3469. [PubMed: 18501424]
64. Du JZ, Du XJ, Mao CQ, Wang J. *J Am Chem Soc*. 2011; 133:17560. [PubMed: 21985458]
65. Odian, G. *Principles of Polymerization*. Wiley; Hoboken, New Jersey: 2004.
66. Iha RK, Wooley KL, Nystrom AM, Burke DJ, Kade MJ, Hawker CJ. *Chem Rev*. 2009; 109:5620. [PubMed: 19905010]
67. Nederberg F, Connor EF, Moeller M, Glauser T, Hedrick JL. *Angew Chem Int Ed*. 2001; 40:2712.
68. Hoyle CE, Lee TY, Roper T. *J Polym Sci, Part A: Polym Chem*. 2004; 42:5301.
69. Zou J, Hew CC, Themistou E, Li Y, Chen CK, Alexandridis P, Cheng C. *Adv Mater*. 2011; 23:4274. [PubMed: 22039596]
70. Fischer D, Li YX, Ahlemeyer B, Kriegelstein J, Kissel T. *Biomaterials*. 2003; 24:1121. [PubMed: 12527253]
71. Dekie L, Toncheva V, Dubrue P, Schacht EH, Barrett L, Seymour LW. *J Control Release*. 2000; 65:187. [PubMed: 10699280]
72. Inkinen S, Hakkarainen M, Albertsson AC, Sodergard A. *Biomacromolecules*. 2011; 12:523. [PubMed: 21332178]
73. Hakkarainen M, Albertsson AC, Karlsson S. *Polym Degrad Stabil*. 1996; 52:283.
74. Yu Y, Zou J, Yu L, Ji W, Li Y, Law WC, Cheng C. *Macromolecules*. 2011; 44:4793.
75. Wang YH, Zheng M, Meng FH, Zhang J, Peng R, Zhong ZY. *Biomacromolecules*. 2011; 12:1032. [PubMed: 21332180]
76. Kim E, Jung Y, Choi H, Yang J, Suh JS, Huh YM, Kim K, Haam S. *Biomaterials*. 2010; 31:4592. [PubMed: 20206379]
77. Takeshita F, Minakuchi Y, Nagahara S, Honma K, Sasaki H, Hirai K, Teratani T, Namatame N, Yamamoto Y, Hanai K, Kato T, Sano A, Ochiya T. *Proc Natl Acad Sci USA*. 2005; 102:12177. [PubMed: 16091473]
78. Singh RK, Lokeshwar BL. *Mol Cancer*. 2009; 8:1476.
79. Sonawane ND, Szoka FC, Verkman AS. *J Biol Chem*. 2003; 278:44826. [PubMed: 12944394]
80. Akinc A, Thomas M, Klibanov AM, Langer R. *J Gene Med*. 2005; 7:657. [PubMed: 15543529]
81. Bertrand N, Leroux JC. *J Control Release*. 2012; 161:152. [PubMed: 22001607]
82. Putnam DA. *Nature Mater*. 2006; 5:439. [PubMed: 16738681]
83. Falamarzian A, Xiong XB, Uludag H, Lavasanifar A. *J Drug Del Sci Tech*. 2012; 22:43.
84. Chomczynski P, Sacchi N. *Anal Biochem*. 1987; 162:156. [PubMed: 2440339]
85. Shively L, Chang L, LeBron JM, Liu Q, Riggs AD, Singer-Sam J. *Biotechniques*. 2003; 34:498. [PubMed: 12669699]
86. Mahajan SD, Schwartz SA, Nair MP. *Biol Proced Online*. 2003; 5:90. [PubMed: 12734551]

Figure 1. 500 MHz ^1H NMR spectra of (a) allyl-functionalized PLA **2**, and (b) CPLA-30 in CDCl_3 .

Figure 2.
GPC curves of allyl-functionalized PLA **2** and CPLAs.

Figure 3.

GPC monitoring of degradation of CPLAs in aqueous solutions: (a) CPLA-18 at 37 °C, (b) CPLA-30 at 37 °C, (c) CPLA-50 at 37 °C, (d) CPLA-18 at 25 °C, (e) CPLA-30 at 25 °C, and (f) CPLA-50 at 25 °C.

Figure 4. Cytotoxicity assessment of CPLAs (PC3 cells were treated with different concentrations of CPLA-18, CPLA-30 and CPLA-50 and incubated for 48 h).

Figure 5. (a) $D_{h,v}$ histogram of the CPLA-50-IL8 siRNA nanoplex in de-ionized water by DLS measurement; (b) TEM image of the CPLA-50-IL8 siRNA nanoplex (TEM samples on carbon films were stained by RuO_4).

Figure 6. Transfection effects of the CPLA-IL-8 siRNA nanoplexes in the PC3 cells for IL-8 gene suppression (TAI: Transcript accumulation index; 48 h of incubation).

Figure 7. Confocal images illustrating the internalization of CPLA-50-IL-8 siRNA^{Cy5} in the PC3 cells (controls: free CPLA-50 and naked IL-8 siRNA^{Cy5}; 4 h of incubation).

Scheme 1.
Conceptual diagram of gene delivery using CPLA.

Scheme 2. Synthesis of CPLA via ROP and thiol-ene functionalization

Table 1Synthesis of CPLAs by thiol-ene functionalization of allyl-functionanlized PLA **2**.^[a]

Entry	[ene] ₀ : [SH] ₀ : [DMPA] ₀	Mol% of amine ^[b]	<i>M</i> _n ^{GPC} ^[c] (kDa)	<i>PD</i> ^{GPC} ^[c]
CPLA-9	1:0.25:0.1	9	12.1	1.20
CPLA-18	1:0.5:0.2	18	11.9	1.21
CPLA-30	1:0.9:0.3	30	11.0	1.23
CPLA-50	1:3:0.4	50	10.5	1.23

^[a] All of the reactions were conducted in CDCl₃ under UV irradiation ($\lambda = 365$ nm) for 30 min; the precursor polymer **2** (*M*_n^{GPC} = 12.0 kDa, *PD*^{GPC} = 1.16) had 50 mol% of allyl group.

^[b] By ¹H NMR spectroscopy.

^[c] Relative to linear polystyrenes.