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Multifunctional Envelope-Type siRNA Delivery Nanoparticle Platform for Prostate Cancer Therapy

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Abstract

With the capability of specific silencing of target gene expression, RNA interference (RNAi) technology is emerging as a promising therapeutic modality for the treatment of cancer and other diseases. One key challenge for the clinical applications of RNAi is the safe and effective delivery of RNAi agents such as small interfering RNA (siRNA) to a particular nonliver diseased tissue (*e.g.*, tumor) and cell type with sufficient cytosolic transport. In this work, we proposed a multifunctional envelope-type nanoparticle (NP) platform for prostate cancer (PCa)-specific *in vivo* siRNA delivery. A library of oligoarginine-functionalized and sharp pH-responsive polymers was synthesized and used for self-assembly with siRNA into NPs with the features of long blood circulation and pH-triggered oligoarginine-mediated endosomal membrane penetration. By further modification with ACUPA, a small molecular ligand specifically recognizing prostate-specific membrane antigen (PSMA) receptor, this envelope-type nanoparticle platform with multifunctional properties can efficiently target PSMA-expressing PCa cells and silence target gene expression. Systemic delivery of the siRNA NPs can efficiently silence the expression of prohibitin 1 (PHB1), which is upregulated in PCa and other cancers, and significantly inhibit PCa tumor growth. These results suggest that this multifunctional envelope-type nanoparticle platform could become an effective tool for PCa-specific therapy.

Graphical abstract

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ASSOCIATED CONTENT

Supporting Information

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Synthesis and characterization of the polymers, PSMA expression on various cancer cells, histology, body weight change of mice, *etc.* (PDF)

The authors declare no competing financial interest.

Keywords

multifunctional nanoparticle; pH-responsive; membrane-penetrating; targeted delivery; siRNA; prostate cancer

Prostate cancer (PCa) is one of the leading causes of cancer death in males worldwide.¹ While localized PCa can be effectively treated by surgery/radiotherapy, many patients experience local cancer recurrence and metastasis.² Androgen deprivation therapy (ADT) is currently the treatment of choice for metastatic PCa and can result in remissions initially. However, resistance develops in most cases, and these patients progress to more aggressive metastatic castration-resistant PCa (mCRPC).^{3,4} Chemotherapeutic drugs, such as docetaxel and recently approved cabazitaxel, abiraterone, and enzalutamide, have been widely used for the treatment of mCRPC,⁵ but they show only moderate survival benefits, and the development of drug resistance has emerged as a persistent clinical problem, limiting their chemotherapy efficacy.⁶ Therefore, there is a critical need for alternative strategies for more effective treatment of advanced PCa.

RNA interference (RNAi) has demonstrated the potential to make a huge impact on disease treatment by silencing the expression of target gene(s) of interest, including those that encode “undruggable” proteins.^{7–10} Over the past decade, nanoparticle (NP)-mediated RNAi therapy has been demonstrated as a powerful method toward this end,^{11–14} especially showing hepatocyte-specificity in nonhuman primates and clinical trials.^{7,15} Nevertheless, the systemic delivery of RNAi agents such as small interfering RNA (siRNA) to a particular nonliver diseased tissue (*e.g.*, solid tumor) and cell type, followed by sufficient intracytosolic transport, has remained a barrier to widespread utilization of RNAi therapeutics. While several RNAi NP platforms have entered into early phase clinical trials for cancer treatment,^{14,16–18} substantial obstacles still remain, including long systemic circulation, selective accumulation at tumor site, and efficient tumor cell internalization and endosomal membrane penetration. Specifically, these challenges are amplified by the unique physiological parameters of certain organs, such as prostate tissues with low microvascular density (MVD) and very slow blood flow rate.^{19,20}

To address these hurdles, we herein developed a multifunctional envelope-type siRNA delivery NP platform for PCa therapy (Scheme 1). These NPs are composed of sharp pH-responsive copolymers containing membrane-penetrating oligoarginine grafts and an *S,S*-2-[3-[5-amino-1-carboxypentyl]-ureido]pentanedioic acid (ACUPA) ligand that can specifically bind to prostate specific membrane antigen (PSMA) overexpressed in advanced

PCa.^{21–23} The resulting polymer/siRNA nanoassembly shows the following functions: (i) the hydrophilic poly(ethylene glycol) (PEG) shells allow the NPs to escape immunological recognition, thus improving blood circulation;^{24–26} (ii) the surface-encoded ACUPA moieties endow the NPs with high PCa specificity and selectivity;^{27,28} (iii) a small population of cationic membrane-penetrating oligoarginine grafts randomly dispersed in the hydrophobic poly(2-(diisopropylamino)ethyl methacrylate) (PDPA) segment can strongly entrap siRNA in the NP core; and (iv) the rapid protonation of the sharp pH-responsive PDPA segment with a pK_a close to endosomal pH (6.0–6.5) causes the fast disassembly of the delivery system,^{29–31} and thus the exposed oligoarginine grafts can penetrate the endosomal membrane for efficient transport of siRNA into the cytoplasm to induce gene silencing.^{32–34} As a proof of concept, we chose prohibitin 1 (PHB1) as a therapeutic target and evaluate the ability of this siRNA delivery system to silence PHB1 expression in PCa. PHB1 is a 32 kDa protein that regulates various cell behaviors such as proliferation, apoptosis, and transcription.^{35–37} Upregulation of PHB1 is observed in different cancers including PCa and associated with drug resistance, and thus PHB1 has been considered a putative cancer biomarker and therapeutic target.^{38–41} However, validation of PHB1 as a potential therapeutic target for PCa treatment remains elusive. In this work, we demonstrate that the multifunctional envelope-type NP platform developed herein can specifically deliver siRNA to PCa cells, silence PHB1 expression, and thus inhibit PCa tumor growth.

RESULTS AND DISCUSSION

Synthesis and Characterization of Multifunctional Polymers and RNAi NPs

Atom-transfer radical polymerization (ATRP) was employed to synthesize the PEGylated polymer methoxyl-polyethylene glycol-*b*-poly(2-(diisopropylamino) ethyl methacrylate-*co*-glycidyl methacrylate) (Meo-PEG-*b*-P(DPA-*co*-GMA)) (Scheme S1). The epoxy group was subsequently subjected to conjugation with oligoarginine (R_n , $n = 6, 8, 10, 20, 30$) to endow the resulting polymer (Meo-PEG-*b*-P(DPA-*co*-GMA- R_n), Scheme 1) with siRNA-loading and endosomal membrane-penetrating abilities. The PSMA-expressing PCa-specific PEGylated polymer ACUPA-PEG-*b*-PDPA (Scheme 1) was also prepared by ATRP, followed by conjugation with ACUPA (Scheme S4). These polymers are characterized by gel permeation chromatography and nuclear magnetic resonance (Figures S1–S3), and the results confirm successful polymer synthesis.

We then varied the length of the oligoarginine grafts to adjust the siRNA loading ability and physiochemical properties of the polymeric NPs, which were prepared by mixing an siRNA aqueous solution with the tetrahydrofuran (THF) solution of Meo-PEG-*b*-P(DPA-*co*-GMA- R_n) at an N/P molar ratio of 80:1. The amphiphilic nature of the polymers induces self-assembly into NPs with siRNA entrapped in the hydrophobic cores. As the number of arginine residues increases from $n = 6$ to 30, the size of the resulting NPs (denoted NPs_{Rn}) increases from 56.6 nm to 189.9 nm (Figure 1A, Table S1), while siRNA encapsulation efficiency (EE%) decreases from 90.6% to 49.7% (Figure 1B). One possible reason is that enhancing the whole hydrophilicity of the amphiphilic polymers by increasing the length of the oligoarginine grafts leads to the formation of looser NPs with weaker siRNA-loading ability, which is supported by the zeta potential of the siRNA-loaded NPs. As shown in

Figure 1B, at the same N/P ratio, the decreased siRNA EE% results in an increased zeta potential. Notably, there is no obvious change in the EE% or size of the NPs made with the mixture of Meo-PEG-*b*-P(DPA-*co*-GMA-Rn) and ACUPA-PEG-*b*-PDPA (denoted ACUPA-NPs_{Rn}, Table S2).

We next chose the amphiphilic polymer Meo-PEG-*b*-P(DPA-*co*-GMA-R10) with a pK_a of 6.31 (Figure S4) to investigate its pH sensitivity. As shown in the transmission electron microscope (TEM) image (Figures 1C and S5), this amphiphilic copolymer can assemble with siRNA to form spherical NPs at a pH of 6.5, with an average size of 90.8 nm determined by dynamic light scattering (DLS, Figure 1A). When the solution pH decreases to 6.0, there are no observable NPs after 20 min of incubation (Figure 1D), indicating a pH-mediated fast NP disassembly. To further evaluate the pH sensitivity, a near-infrared dye Cy5.5-conjugated PEGylated polymer (Scheme S3) was mixed with Meo-PEG-*b*-P(DPA-*co*-GMA-R10) to prepare the NPs with the entrapment of fluorophores inside the hydrophobic cores. Due to the self-quenching of the entrapped fluorophores, the fluorescence signal is very weak at a pH of 6.5 or 7.0 (Figure S6). However, protonation of the PDPA segment at a pH below pK_a causes the NPs to disassemble, leading to a dramatic increase in the fluorescence signal. Measuring the fluorescence intensity upon the pH change reveals that the pH difference from 10% to 90% fluorescence activation ($pH_{10-90\%}$) is 0.39 (Figure S7).⁴² This value is much smaller than that of small-molecule dyes such as aminophenyl BODIPY (about 2 pH units) with the same degree of fluorescence intensity change,⁴³ demonstrating the fast pH response rate of Meo-PEG-*b*-P(DPA-*co*-GMA-R10). This characteristic allows the NPs of this polymer to show a pH-dependent release of DY547-labeled luciferase (Luc) siRNA (DY547-siRNA) at a pH below pK_a . As shown in Figure 1E, around 80% of the loaded siRNA has been released within 3 h at a pH of 6.0. Within the same time frame, less than 30% of the loaded siRNA is released at a pH of 7.4.

***In Vitro* Gene Silencing**

Firefly luciferase-expressing HeLa (Luc-HeLa) cells were employed to evaluate the gene silencing efficacy of the siRNA-loaded NPs. The Luc siRNA was used to selectively suppress firefly Luc expression. As shown in Figure 2A, all the siRNA-loaded NPs can suppress the Luc expression at a 10 nM siRNA dose, with differential silencing efficacy depending on the length of the oligoarginine grafts. Note that there is no obvious difference between the NPs with and without the ACUPA ligand for Luc silencing due to the extremely low PSMA expression in HeLa cells (Figure S8), which leads to similar cellular uptake between these two types of NPs (Figure 2B). Among these nanoplateforms, the NPs self-assembled from Meo-PEG-*b*-P(DPA-*co*-GMA-R8) or Meo-PEG-*b*-P(DPA-*co*-GMA-R10) show a better gene silencing efficacy. In particular, the NPs made with Meo-PEG-*b*-P(DPA-*co*-GMA-R10) can reduce the luciferase expression by about 90%, better than the commercial Lipofectamine 2000 (Lipo2K) treatment, that shows around 80% knockdown in Luc expression. Notably, there is no obvious cytotoxicity of the NPs used for these *in vitro* transfection experiments (Figure S9).

To validate that the efficient silencing efficacy of the NPs prepared from Meo-PEG-*b*-P(DPA-*co*-GMA-R10) (NPs_{R10}) is attributable to their excellent endosomal escape

capability, we used LysoTracker green to label the endosomes and examined the intracellular distribution of the siRNA-loaded NPs. The confocal laser scanning microscope (CLSM) image in Figure 2C indicates efficient cytosolic siRNA delivery after 2 h of incubation with siRNA-loaded NPs at physiological temperature (37 °C). When decreasing the incubation temperature to 4 °C, at which endocytic processes are arrested, the amount of internalized NPs is extremely low, as demonstrated by the very weak fluorescence (Figure S10). This result suggests that the cellular uptake of the siRNA-loaded NPs is energy dependent. If the R10 grafts are replaced by tetraethylenepentamine without membrane-penetrating function (Meo-PEG-*b*-P(DPA-*co*-GMA-TEPA), Scheme S2), the endosomal escape ability of the resulting NPs is comparatively weaker (Figure 2D), leading to a much lower silencing efficacy (Figure S11). This highlights the importance of the oligoarginine-mediated endosomal membrane penetration for siRNA transport into cytosols. Additionally, the better silencing efficacy of NPs_{R8} and NPs_{R10} also agrees with the contention that the length of oligoarginine for the most efficient membrane penetration is between 8 and 10 arginine residues.^{44–47}

After screening the nanoplateform with optimal silencing efficacy (NPs_{R10}), we then evaluated its ability to protect siRNA from RNase degradation and deliver siRNA to target PCa. Fluorescein- and its quencher (Dabcyl)-labeled siRNA were encapsulated into the NPs to examine their ability to protect siRNA.⁴⁸ When incubating with the RNase, naked siRNA can be rapidly degraded within 15 min, which induces the dissociation between fluorescein and its quencher, and thereby significant increase the fluorescence intensity (Figure S12). However, there is nearly no fluorescence change after 4 h of incubation of the siRNA-loaded NPs_{R10} with RNase, demonstrating the strong ability of this NP platform to protect the siRNA from degradation. LNCaP cells, a PCa cell line with overexpressed PSMA (Figure S8),⁵ were chosen for incubation with the DY547-siRNA-loaded NPs to assess their PCa-targeting ability. From the flow cytometry profile displayed in Figure 3A, unlike the Luc-HeLa cells, LNCaP cells show much higher internalization of DY547-siRNA-loaded ACUPA-NPs_{R10}, and the intracellular fluorescence intensity is around 5-fold stronger than that of the cells incubated with siRNA-loaded NPs_{R10} (Figure S13). If the cells are pretreated with free ACUPA or PSMA antibody, there is no obvious difference in cellular uptake between ACUPA-NPs_{R10} and NPs_{R10}, indicating that the targeted uptake of ACUPA-NPs_{R10} is built on the specific recognition between the ACUPA ligand and the overexpressed PSMA on LNCaP cells. To further validate this ACUPA-mediated PCa-targeting ability, two other PCa cell lines with extremely low PSMA expression, PC3 and DU145 cells (Figure S8), were also incubated with the DY547-siRNA-loaded NPs. With the absence of a specific interaction between the ACUPA ligand and PSMA, the cells show a similar ability to internalize the ACUPA-NPs_{R10} and NPs_{R10} (Figure 3B and C), and there is no difference in the intracellular fluorescence intensity (Figure S13).

On the basis of the strong PCa-targeting ability of ACUPA-NPs_{R10}, we then examined whether this siRNA delivery nanoplateform can be used to silence PHB1 in LNCaP cells. PHB1 is upregulated in different cancers including PCa, and the biological function depends on its cellular localization.^{35,39} PHB1 is mainly located in the cytoplasm and plays an important role in the maintenance of mitochondrial function and protection against senescence.³⁵ Silencing PHB1 expression in the cytoplasm shows the ability to induce

mitochondrion dysfunction and inhibit cancer cell proliferation.⁴¹ Besides in the cytoplasm, PHB1 is also found on the cell membrane and in the nucleus. Cell membrane PHB1 is related to drug resistance, and its downregulation can improve the sensitivity of cancer cells to chemotherapeutic drugs.⁴⁰ For the PHB1 located in the nucleus, it shows a different function compared to the one in the cytoplasm and serves as a tumor suppressor *via* interaction with p53 and retinoblastoma protein (pRb).^{49–51} Prior to evaluating the silencing efficacy of the siRNA-loaded NPs in LNCaP cells, we first examined PHB1 expression using immunofluorescence. The CLSM image in Figure 3D shows that the majority of PHB1 is located in the cytoplasm with a very small amount in the nucleus, which is consistent with a previous report that LNCaP cells show the localization of PHB1 in both the cytoplasm and nucleus.⁴⁹ Figure 3E and F show the PHB1 expression in LNCaP cells treated by the PHB1 siRNA-loaded NPs. Red fluorescence corresponding to residual PHB1 expression can still be observed in the LNCaP cells treated by siRNA-loaded NPs_{R10} at a 10 nM siRNA dose (Figure 3E). In contrast, there is much weaker red fluorescence in the cells treated by siRNA-loaded ACUPA-NPs_{R10} (Figure 3F). A similar result can also be found in the Western blot analysis. This siRNA delivery nanoplatform can silence PHB1 at a 10 nM siRNA dose (Figures 3G and S14). Additionally, the PHB1 expression is nearly absent at a 50 nM siRNA dose. For comparison, more PHB1 is still expressed in the cells incubated with the siRNA-loaded NPs_{R10} without ACUPA targeting. With this suppressed PHB1 expression to inhibit the proliferation of LNCaP cells,³⁵ the cell number shows only roughly a 3-fold increase after 8 days of incubation with the siRNA-loaded ACUPA-NPs_{R10} at a 10 nM siRNA dose (Figure 3H). In contrast, there is a 6.7-fold or 10.7-fold increase in the number of cells after treatment with PHB1 or Luc siRNA-loaded NPs_{R10}.

Pharmacokinetics and Biodistribution

After validation of the *in vitro* PCa-selectivity of the ACUPA-NPs_{R10}, we then evaluated their pharmacokinetics and *in vivo* PCa-targeting ability. The pharmacokinetics of the ACUPA-NPs_{R10} was examined by intravenous injection of DY647-labeled Luc siRNA (DY647-siRNA) loaded NPs to healthy mice (1 nmol of siRNA dose in 200 μ L of PBS buffer, $n = 3$). As shown in Figure 4A, the circulation half-life ($t_{1/2}$) of NPs_{R10} in blood is around 4.57 h, which is comparable to the $t_{1/2}$ of ACUPA-NPs_{R10} (~4.76 h) but far longer than naked siRNA ($t_{1/2} < 10$ min). This longer circulation feature is mainly attributed to protection by the PEG outer layer.^{24–26} The *in vivo* PCa-targeting ability of ACUPA-NPs_{R10} was assessed by intravenously injecting DY677-labeled Luc siRNA (DY677-siRNA)-loaded NPs to LNCaP xenograft tumor-bearing mice (1 nmol of siRNA dose in 200 μ L of PBS buffer, $n = 3$). Figure 4B shows the fluorescent image of the mice 24 h postinjection. There is a very weak accumulation of naked siRNA in the tumor. In comparison, the ACUPA-NPs_{R10} show high accumulation in the tumor corresponding to the bright fluorescence. In the absence of the PSMA-specific ACUPA ligand, the accumulation of NPs_{R10} in the tumor is much lower compared to ACUPA-NPs_{R10}. If first injecting the PSMA antibody (5 mg/kg dose) followed by ACUPA-NPs_{R10}, the PSMA blocking led to a decrease of the tumor accumulation of ACUPA-NPs_{R10}, highlighting the important effect of a specific interaction between PSMA and the ACUPA ligand on the PCa-targeting ability of the NPs. To analyze the biodistribution of NPs, we harvested the tumor and main organs of mice 24 h postinjection (Figure 4C), and the biodistribution of the NPs in each organ is shown in

Figure 4D. The naked siRNA presents a characteristic biodistribution, *i.e.*, high accumulation in kidney but extremely low accumulation in tumor.⁴¹ With the specific recognition between the ACUPA ligand and PSMA overexpressed on LNCaP xenograft tumor, the accumulation of ACUPA-NPs_{R10} in tumor is around 3-fold higher than that of NPs_{R10} or that found in mice pretreated with PSMA antibody.

In Vivo Gene Silencing and Anti-Tumor Efficacy

To examine the inhibition of PHB1 expression in tumor tissue, PHB1 siRNA-loaded NPs were intravenously injected into LNCaP xenograft tumor-bearing mice (1 nmol of siRNA dose in 200 μ L of PBS buffer, $n = 3$) on three consecutive days, and *in vivo* PHB1 expression was examined by Western blot. Before the injection, endotoxin concentration was examined using the gel clot LAL assay (Figure S15). Results show the endotoxin concentration is lower than 0.0625 EU/mL in the solution of both PHB1 siRNA-loaded NPs_{R10} and ACUPA-NPs_{R10}. In this work, the body weight of mice we used is around 25 g, and the endotoxin dose in each injection can be calculated as <0.5 EU/kg, which is a safe dose and 10-fold lower than the FDA's limit (5 EU/kg) for the allowable amount of endotoxin in drug formulations.⁵² Figure 5A and B show the PHB1 expression in the tumor tissue of the LNCaP xenograft tumor-bearing mice. With PSMA targeting, ACUPA-NPs_{R10} led to around 70% knockdown of PHB1 expression. In contrast, there is only around 37% knockdown for mice treated with siRNA-loaded NPs_{R10}. A similar tendency can also be found in the immunohistochemistry (IHC) staining analysis (Figure 5D and E). With the ACUPA-mediated LNCaP tumor targeting, the PHB1 expression in the ACUPA-NPs_{R10} group is much lower than that of the NPs_{R10} group. Notably, the administration of the siRNA-loaded NPs shows negligible *in vivo* side effects. There is no obvious change of TNF- α , IFN- γ , IL-6, and IL-12 level in serum 24 h postinjection of the siRNA-loaded NPs (Figure S16). After three consecutive injections of the NPs to healthy mice (once every 2 days at a 1 nmol siRNA dose in 200 μ L of PBS buffer, $n = 3$), there are no noticeable histological changes in the tissues from heart, liver, spleen, lung, or kidney (Figure S17). To evaluate whether this NP-mediated PHB1 silencing has an antitumor effect, the PHB1 siRNA-loaded NPs were intravenously injected into the LNCaP xenograft tumor-bearing mice (1 nmol of siRNA dose in 200 μ L of PBS buffer, $n = 5$). As shown in Figure 5F–H, the siRNA-loaded NPs can inhibit tumor growth. In particular, due to their high PCa selectivity, the siRNA-loaded ACUPA-NPs_{R10} significantly suppress tumor growth after five consecutive injections with less than 3-fold increase in the tumor size at day 30. For the mice treated with Luc siRNA-loaded NPs_{R10} (control NPs) or PBS, a more than 6-fold or 8-fold increase in the tumor size can be found at 18 days after the first injection. Moreover, the siRNA-loaded ACUPA-NPs_{R10} shows no obvious influence on mouse body weight (Figure S18), implying good biocompatibility of this nanoplatform.

CONCLUSIONS

We have developed an oligoarginine-functionalized and sharp pH-responsive NP platform for targeted PCa siRNA delivery. This targeted nanoplatform can deliver siRNA to PCa through the specific recognition between the ACUPA ligand and overexpressed PSMA on PCa cells. With the oligoarginine-mediated membrane penetration, these NPs can efficiently

escape from endosomes and rapidly transport therapeutic siRNA to the cytoplasm, leading to a significant inhibition of cancer-associated PHB1 expression and LNCaP tumor growth. This multifunctional envelope-type NP system reported here shows great potential as a robust siRNA delivery vehicle for PCa-specific therapy.

METHODS

Preparation and Characterization of NPs

Meo-PEG-*b*-P(DPA-*co*-GMA-Rn) was dissolved in THF to form a homogeneous solution with a concentration of 4 mg/mL. Subsequently, a certain volume of this THF solution was taken and mixed with 1 nmol of siRNA (0.1 nmol/ μ L aqueous solution) in an N/P molar ratio of 80:1. Under vigorous stirring (1000 rpm), the mixture was added dropwise to 4 mL of deionized water. The NP dispersion formed was transferred to an ultrafiltration device (EMD Millipore, MWCO 100 K) and centrifuged to remove the organic solvent and free compounds. After washing with phosphate-buffered saline (PBS, pH 7.4) solution (3×5 mL), the siRNA-loaded NPs were dispersed in 1 mL of PBS buffer. Size and zeta potential were determined by DLS (Brookhaven Instruments Corporation). The morphology of NPs was visualized by transmission electron microscopy (TEM, Tecnai G² Spirit BioTWIN). To determine the siRNA EE%, DY547-labeled Luc siRNA (DY547-siRNA)-loaded NPs were prepared according to the method aforementioned. A small volume (5 μ L) of the NP solution was withdrawn and mixed with 20-fold DMSO. The standard was prepared by mixing 5 μ L of naked DY547-siRNA solution (1 nmol/mL) with 20-fold DMSO. The fluorescence intensity of DY547-siRNA was measured using a Synergy HT multimode microplate reader (BioTek Instruments), and the siRNA EE% is calculated as $EE\% = (FI_{NPs}/FI_{Standard}) \times 100$.

To prepare the ACUPA-NPs, Meo-PEG-*b*-P(DPA-*co*-GMA-Rn) (4 mg/mL in THF) was mixed with 1 nmol of siRNA (0.1 nmol/ μ L aqueous solution) in an N/P molar ratio of 80:1. Then ACUPA-PEG-*b*-PDPA (4 mg/mL in THF, 10 mol % compared to Meo-PEG-*b*-P(DPA-*co*-GMA-Rn)) was added, and the mixture was added dropwise to 4 mL of deionized water. The ACUPA-NPs were purified, and siRNA EE% was determined according to the method aforementioned.

In Vitro siRNA Release

DY547-siRNA-loaded NPs were dispersed in 1 mL of PBS (pH 7.4) and then transferred to a Float-a-lyzer G2 dialysis device (MWCO 100 kDa, Spectrum) that was immersed in PBS (pH 7.4) at 37 °C. At a predetermined interval, 5 μ L of the NP solution was withdrawn and mixed with 20-fold DMSO. The fluorescence intensity of DY547-siRNA was determined by a Synergy HT multimode microplate reader.

Cell Culture

Luc-HeLa and PCa cell lines (LNCaP, PC3, DU145, 22RV1) were incubated in RPMI 1640 medium with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO₂.

Luciferase Silencing

Luc-HeLa cells were seeded in 96-well plates (5000 cells per well) and incubated in 0.1 mL of RPMI 1640 medium with 10% FBS for 24 h. Thereafter, the Luc siRNA-loaded NPs were added. After incubating for 24 h, the cells were washed with fresh medium and allowed to incubate for another 48 h. The Luc expression was determined using Steady-Glo luciferase assay kits. Cytotoxicity was measured using the AlamarBlue assay according to the manufacturer's protocol. The luminescence or fluorescence intensity was measured using a microplate reader, and the average value of five independent experiments was collected.

Endosomal Escape

Luc-HeLa cells (20 000 cells) were seeded in discs and incubated in 2 mL of RPMI 1640 medium containing 10% FBS for 24 h. Subsequently, the DY547-siRNA-loaded NPs were added, and the cells were allowed to incubate for 2 h at 37 and 4 °C. After removing the medium and subsequently washing with PBS buffer thrice, the endosomes and nuclei were stained with Lysotracker green and Hoechst 33342, respectively. The cells were then viewed under a FV1000 CLSM (Olympus).

Flow Cytometry

Luc-HeLa and PCa cell lines (LNCaP, PC3, DU145) were seeded in six-well plates (50 000 cells per well) and incubated in 2 mL of RPMI 1640 medium containing 10% FBS for 24 h. Subsequently, the DY547-siRNA-loaded NPs or ACUPA-NPs were added, and the cells were allowed to incubate for another 4 h. After removing the medium and subsequently washing with PBS buffer thrice, the cells were collected for flow cytometry.

Digestion Assay

NPs loaded with fluorescein and its quencher (Dabcyl)-labeled Luc siRNA were prepared according to the method aforementioned and then dispersed in 1 mL of PBS buffer. Subsequently, 20 U RNase was added, and the sample was incubated in 37 °C. At predetermined time intervals, the fluorescent emission spectra were examined using a plate reader with excitation at 480 nm and emission data range between 490 and 650 nm.

In Vitro PHB1 Silencing

LNCaP cells were seeded in six-well plates (50 000 cells per well) and incubated in 2 mL of RPMI 1640 medium containing 10% FBS for 24 h. Subsequently, the cells were incubated with the PHB1 siRNA-loaded NPs or ACUPA-NPs for 24 h. After washing the cells with PBS buffer thrice, the cells were further incubated in fresh medium for another 48 h. Thereafter, the cells were digested by trypsin and the proteins were extracted using modified radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40 substitute, 0.25% sodium deoxycholate, 1 mM sodium fluoride, 1 mM Na₃VO₄, 1 mM EDTA), supplemented with protease inhibitor cocktail and 1 mM phenylmethanesulfonyl fluoride (PMSF). The PHB1 expression was examined by using Western blot.

Western Blot

Equal amounts of proteins were added to SDS-PAGE gels and separated by gel electrophoresis. After transferring the proteins from gel to polyvinylidene difluoride (PVDF) membrane, the blots were blocked with 3% BSA in TBST (50 mM Tris-HCl pH 7.4, 150 mM NaCl, and 0.1% Tween 20) and then incubated with a mixture of PHB1 rabbit antibody (Cell Signaling) and β -actin rabbit antibody (Cell Signaling). The PHB1 expression was detected with horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit IgG HRP-linked antibody, Cell Signaling) and an enhanced chemiluminescence (ECL) detection system (Pierce).

Immunofluorescence Staining

LNCaP cells (50 000 cells) were seeded in a round disc and incubated in 2 mL of RPMI1640 medium containing 10% FBS for 24 h. After PHB1 silencing with PHB1 siRNA-loaded NPs or ACUPA-NPs, the cells were fixed with 4% paraformaldehyde. The cells were then permeabilized by incubation in 0.2% Triton X-100 in PBS for 5 min, followed by washing with PBS (3 $\times$ 5 min). Thereafter, the cells were blocked with blocking buffer (2% normal goat serum, 2% BSA, and 0.2% gelatin in PBS) at room temperature for 1 h. After washing the cells with PBS (3 $\times$ 5 min), PHB1 rabbit antibody (Abcam) diluted in 1% BSA solution was added and the cells were incubated for 1 h. Subsequently, PBS buffer (3 $\times$ 5 min) was added to the cells, which were then further incubated with Alex Fluoro 647-linked secondary antibody and Alex Fluoro 488-conjugated phalloidin for another 1 h. After washing with PBS buffer (3 $\times$ 5 min), the cells were stained with Hoechst 33342 and finally viewed under a FV1000 CLSM (Olympus).

In Vitro Inhibition of Cell Proliferation

LNCaP cells were seeded in six-well plates (20 000 cells per well) and incubated in 2 mL of RPMI 1640 medium containing 10% FBS for 24 h. Thereafter, the cells were incubated with the PHB1 siRNA-loaded NPs or ACUPA-NPs for 24 h and then washed with fresh medium for further incubation. At predetermined intervals, the cytotoxicity was measured using the AlamarBlue assay according to the manufacturer's protocol. After each measurement, the AlamarBlue agent was removed, and the cells were incubated in fresh medium for further proliferation.

Animals

Healthy male BALB/c normal mice and Athymic nude mice (4–5 weeks old) were purchased from Charles River Laboratories. All *in vivo* studies were performed in accordance with National Institutes of Health animal care guidelines and in strict pathogen-free conditions in the animal facility of Brigham and Women's Hospital. The animal protocol was approved by the Institutional Animal Care and Use Committees on animal care (Harvard Medical School).

LNCaP Xenograft Tumor Model

The tumor model was constructed by subcutaneous injection with 200 μ L of LNCaP cell suspension (a mixture of RPMI 1640 medium and Matrigel in 1:1 volume ratio) with a

density of 2×10^6 cells/mL into the back region of healthy male athymic nude mice. When the volume of the tumor xenograft reached $\sim 50 \text{ mm}^3$, the mice were used for the *in vivo* experiments.

Pharmacokinetics Study

Healthy male BALB/c normal mice were randomly divided into three groups ($n = 3$) and given an intravenous injection of either (i) naked DY647-siRNA, (ii) DY647-siRNA-loaded NPs, or (iii) DY647-siRNA-loaded ACUPA-NPs at a 1 nmol siRNA dose in 200 μL of PBS buffer. At predetermined time intervals, 20 μL of blood was retro-orbitally withdrawn and the wound was pressed for several seconds to stop the bleeding. The fluorescence intensity of DY647-siRNA in the blood was determined using a microplate reader.

Biodistribution

LNCaP tumor-bearing male athymic nude mice were randomly divided into four groups ($n = 3$) and given an intravenous injection of either (i) naked DY677-siRNA, (ii) DY677-siRNA-loaded NPs, (iii) DY677-siRNA-loaded ACUPA-NPs, or (iv) PSMA antibody (5 mg/kg dose) in 15 min followed by DY677-siRNA-loaded ACUPA-NPs at a 1 nmol siRNA dose in 200 μL of PBS buffer. Twenty-four hours after the injection, the mice were imaged using the Maestro 2 In-Vivo imaging system (Cri Inc.). Main organs and tumors were then harvested and imaged. To quantify the accumulation of NPs in tumors and organs, the fluorescence intensity of each tissue was quantified by Image-J.

In Vivo PHB1 Silencing

LNCaP tumor-bearing male athymic nude mice were randomly divided into three groups and intravenously injected with (i) PHB1 siRNA-loaded NPs ($n = 3$), (ii) PHB1 siRNA-loaded ACUPA-NPs ($n = 3$), or (iii) Luc siRNA-loaded NPs ($n = 2$) at a 1 nmol siRNA dose in 200 μL of PBS buffer for three consecutive days. Twenty-four hours after the final injection, the mice were sacrificed and tumors were harvested. The proteins in the tumor were extracted using modified radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40 substitute, 0.25% sodium deoxycholate, 1 mM sodium fluoride, 1 mM Na_3VO_4 , 1 mM EDTA), supplemented with protease inhibitor cocktail and 1 mM PMSF. The PHB1 expression was examined using Western blot.

Immunohistochemistry Staining

Immunohistochemistry staining was performed on formalin-fixed paraffin-embedded tumor sections. Briefly, tumor slides were first heated to 60 $^\circ\text{C}$ for 1 h, desparaffinized with xylene ($3 \times 5 \text{ min}$), and washed with different concentrations of alcohol. After retrieval of antigen using DAKO target retrieval solution at 95–99 $^\circ\text{C}$ for 40 min, followed by washing, the slides were blocked with peroxidase blocking buffer (DAKO Company) for 5 min. After washing the buffer (DAKO Company), the slides were incubated with PHB1 rabbit antibody (Abcam) diluted in DAKO antibody solution for 1 h. The slides were then washed and incubated with peroxidase-labeled polymer for 30 min. After washing and staining with DAB + substrate-chromogen solution and hematoxylin, the slides were remounted and viewed under an MVX10 MacroView dissecting scope equipped with an OlympusDP80 camera.

Immune Response

Healthy male BALB/c normal mice were randomly divided into three groups ($n = 3$) and given an intravenous injection of either (i) PBS, (ii) PHB1 siRNA-loaded NPs, or (iii) PHB1 siRNA-loaded ACUPA-NPs at a 1 nmol siRNA dose in 200 μL of PBS buffer. Serum samples obtained 6 and 24 h postinjection were processed for measurement of representative cytokines (TNF- α , IFN- γ , IL-6, and IL-12) by enzyme-linked immunosorbent assay or ELISA (PBL Biomedical Laboratories and BD Biosciences) in accordance with the manufacturer's instructions.

Inhibition of Tumor Growth

LNCaP tumor-bearing male athymic nude mice were randomly divided into four groups ($n = 5$) and intravenously injected with (i) PBS, (ii) Luc siRNA-loaded NPs, (iii) PHB1 siRNA-loaded NPs, or (iv) PHB1 siRNA-loaded ACUPA-NPs at a 1 nmol siRNA dose in 200 μL of PBS buffer once every 2 days. All the mice were administered five consecutive injections, the tumor growth was monitored every 2 days by measuring perpendicular diameters using a caliper, and tumor volume was calculated as follows:

$$V = W^2 \times L / 2$$

where W and L are the shortest and longest diameters, respectively.

Histology

Healthy male BALB/c normal mice were randomly divided into three groups ($n = 3$) and administered daily intravenous injections of (i) PBS, (ii) PHB1 siRNA-loaded NPs, or (iii) PHB1 siRNA-loaded ACUPA-NPs at a 1 nmol siRNA dose in 200 μL of PBS buffer. After three consecutive injections (once every 2 days), the main organs were collected 2 days after the final injection, fixed with 4% paraformaldehyde, and embedded in paraffin. Tissue sections were stained with hematoxylin-eosin (H&E) and viewed under an optical microscope.

Statistical Analysis

Statistical significance was determined by a two-tailed Student's t test assuming equal variance. A p value of <0.05 is considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterizations of the siRNA-loaded NPs. (A) Size and polydispersity (PDI) of the Luc siRNA-loaded NPs of Meo-PEG-*b*-P(DPA-*co*-GMA-Rn). (B) Zeta potential (ζ) and siRNA EE% of the Luc siRNA-loaded NPs of Meo-PEG-*b*-P(DPA-*co*-GMA-Rn). EE% was determined by examining the fluorescence intensity of DY547-siRNA and then comparing to the fluorescence intensity of a standard solution. (C, D) TEM images of the Luc siRNA-loaded NPs of Meo-PEG-*b*-P(DPA-*co*-GMA-R10) incubated in PBS buffer at a pH of (C) 6.5 and (D) 6.0. (E) *In vitro* release of DY745-siRNA from the NPs of Meo-PEG-*b*-P(DPA-*co*-GMA-R10) at a pH of 6.0 and 7.4.

Figure 2.

Gene silencing and endosomal escape ability of the siRNA-loaded NPs. (A) Luc expression in Luc-HeLa cells transfected with Luc siRNA-loaded NPs of Meo-PEG-*b*-P(DPA-*c*o-GMA-Rn) and Lipo2K-siRNA complex at a 10 nM siRNA dose. Control: Cells incubated with free medium. (B) Flow cytometry profile and mean fluorescence intensity (MFI) of Luc-HeLa cells incubated with DY547-siRNA-loaded NPs_{R10} and ACUPA-NPs_{R10} at 37 °C for 4 h at a 10 nM siRNA dose. Blank: Cells incubated with free medium. (C, D) CLSM images of Luc-HeLa cells incubated with the DY547-siRNA loaded NPs_{R10} (C) and NPs made of the polymer without R10 grafts (D) at 37 °C for 2 h. Endosomes are stained by LysoTracker green; nuclei were stained by Hoechst 33342. (i) DY547-siRNA with red fluorescence; (ii) endosomes with green fluorescence; (iii) nuclei with blue fluorescence; (iv) overlap of (i), (ii), and (iii).

Figure 3.

In vitro PCa-targeting ability of the siRNA-loaded NPs and their gene silencing efficacy in PCa cells. (A–C) Flow cytometry profiles of LNCaP (A), PC3 (B), and DU145 (C) cells incubated with DY547-siRNA-loaded NPs at 37 °C for 4 h at a 10 nM siRNA dose (a, NPs_{R10}; b, ACUPA-NPs_{R10}; c, free ACUPA-pretreated cells incubated with ACUPA-NPs_{R10}; d, PSMA antibody-pretreated cells incubated with ACUPA-NPs_{R10}). (D–F) Immunofluorescence analysis of the LNCaP cells treated by Luc siRNA-loaded NPs_{R10} (D) and PHB1 siRNA-loaded NPs_{R10} (E) and ACUPA-NPs_{R10} (F) at a 10 nM siRNA dose. Red fluorescence indicates PHB1 expression. (G) Western blot analysis of PHB1 expression in LNCaP cells treated with PHB1 siRNA-loaded NPs_{R10} and ACUPA-NPs_{R10} at different

siRNA doses. Luc siRNA-loaded NPs_{R10} (control 1) and ACUPA-NPs_{R10} (control 2) were used as different controls. (H) Proliferation profile of LNCaP cells treated with PHB1 siRNA-loaded NPs_{R10} and ACUPA-NPs_{R10} at a 10 nM siRNA dose. Luc siRNA-loaded NPs_{R10} (control 1) and ACUPA-NPs_{R10} (control 2) were used as controls.

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Figure 4.

Blood circulation and *in vivo* PCa-targeting ability of the siRNA-loaded NPs. (A) Pharmacokinetics of naked DY647-siRNA and DY647-siRNA loaded NPs_{R10} and ACUPA-NPs_{R10}. The naked siRNA and siRNA-loaded NPs were intravenously injected into mice, and then 20 μ L of blood was collected retro-orbitally at different time points. The residual naked siRNA and siRNA-loaded NPs were determined by fluorescence spectroscopy. (B) Overlaid fluorescent image of the LNCaP xenograft tumor-bearing nude mice 24 h postinjection of naked DY677-siRNA, DY677-siRNA-loaded NPs_{R10}, and ACUPA-NPs_{R10} and PSMA antibody followed by DY677-siRNA-loaded ACUPA-NPs_{R10}. (C) Overlaid fluorescent image of the tumors and main organs of the LNCaP xenograft tumor-bearing nude mice sacrificed 24 h postinjection of DY677-siRNA-loaded ACUPA-NPs_{R10} (i) and NPs_{R10} (ii), PSMA antibody followed by DY677-siRNA-loaded ACUPA-NPs_{R10} (iii), and naked DY677-siRNA (iv). (D) Biodistribution of the NPs quantified from (C).

Figure 5.

In vivo gene silencing and antitumor effect of the siRNA-loaded NPs. (A, B) Western blot analysis of PHB1 expression in the LNCaP tumor tissue after systemic treatment by control NPs and PHB1 siRNA-loaded NPs_{R10} and ACUPA-NPs_{R10}. (C–E) Immunohistochemistry analysis of PHB1 expression in the LNCaP tumor tissue after systemic treatment by control NPs (C) and PHB1 siRNA-loaded NPs_{R10} (D) and ACUPA-NPs_{R10} (E). (F) Relative tumor size of the LNCaP xenograft tumor-bearing nude mice ($n = 5$) after treatment by PBS, control NPs, and PHB1 siRNA-loaded NPs_{R10} and ACUPA-NPs_{R10}. The intravenous injections are indicated by the arrows. * $P < 0.05$; ** $P < 0.01$ (G) Representative photograph of the LNCaP xenograft tumor-bearing nude mice in each group at day 18. (H) Photograph of the harvested LNCaP tumors after a 30-day evaluation. Luc siRNA-loaded NPs_{R10} were used as a control.

Scheme 1.

Molecular structures of the oligoargininefunctionalized sharp pH-responsive polymer Meo-PEG-*b*-P(DPA-co-GMA-Rn) and PCa-specific polymer ACUPAPEG-*b*-PDPA and a schematic illustration of the multifunctional envelope-type NP platform for *in vivo* PCa-specific siRNA delivery and therapy. The two polymers can co-assemble with siRNA to form stable NPs with ACUPA targeting ligands encoded on the surface (a). After intravenous administration to mice (b, c), the siRNA-loaded NPs can extravasate from leaky tumor vasculature (d) and target the tumor tissue through the specific interaction between ACUPA and overexpressed PMSA on PCa cells (e). After cellular uptake (f), the sharp pH-responsive characteristics of the polymers induce fast disassembly of the NPs, and the exposed membrane-penetrating oligoarginine grafts lead to efficient endosomal escape (g), thus resulting in efficient gene silencing to inhibit tumor growth (h).