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Delivery of Multiple siRNAs Using Lipid-coated PLGA Nanoparticles for Treatment of Prostate Cancer

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Abstract

Nanotechnology can provide a critical advantage in developing strategies for cancer management and treatment by helping to improve the safety and efficacy of novel therapeutic delivery vehicles. This paper reports the fabrication of poly(lactic acid-*co*-glycolic acid)/siRNA nanoparticles coated with lipids for use as prostate cancer therapeutics made via a unique soft lithography particle

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SUPPORTING INFORMATION AVAILABLE. The stability of lipid-coated particles in PBS and 10% serum/PBS is provided. The release profile of the siRNA from the lipid-coated PLGA particle is also included. This information is available free of charge via the Internet at http://pubs.acs.org/.

molding process called PRINT (Particle Replication In Nonwetting Templates). The PRINT process enables high encapsulation efficiency of siRNA into neutral and monodisperse PLGA particles (32–46% encapsulation efficiency). Lipid-coated PLGA/siRNA PRINT particles were used to deliver therapeutic siRNA *in vitro* to knockdown genes relevant to prostate cancer.

Keywords

Soft lithography; siRNA delivery; PLGA; lipids; PRINT; prostate cancer

Almost 30,000 men die every year with prostate cancer that becomes refractory to androgen deprivation therapy.[1] New therapeutic options and detection strategies are clearly needed. Current research in nanomedicine is driven by the desire to create a methodology to effectively deliver a range of biologically and therapeutically relevant cargos and to improve the therapeutic index. Targeted cancer therapy by RNA interference (RNAi) is an approach that can be used to silence genes *in vivo* by selectively targeting genes such as KIF11 (Eg5), which encodes a motor protein that belongs to the kinesin-like protein family involved in chromosome positioning and bipolar spindle formation during cell mitosis.

Small interfering RNAs (siRNA) are double-stranded RNA molecules approximately 21–25 base pairs (bp) long that act to inhibit gene expression through initiating enzymatic degradation of a sequence-matched mRNA.[2] siRNA-based therapeutic agents may provide a promising way to overcome disease such as cancer by the natural process of RNA interference.[3–5] However, intracellular delivery of siRNA delivery is challenging because siRNA is negatively charged and is prone to degradation in physiological conditions. Therefore a non-toxic and non-immunogenic carrier is required to deliver the siRNA to its action site, which will dramatically improve its clinical potential. An ideal carrier should be able to protect siRNA, transport siRNA to target tissue, be internalized by target cells and release siRNA to cytosol, but induce minimal toxicity or immune response.[6] To address these issues, carriers have been developed to deliver siRNA by combining cationic polymers, peptides, and lipids with siRNA to form polyplexes and lipoplexes. [7–9]

Conventional polyplex and lipoplex formulations are held together electrostatically and by their bilayers; therefore requiring that these oppositely charged molecules remain complexed while extracellularly followed by their disassembly once the complex is internalized into the cell of choice. Both the serum and the extracellular matrix can lead to vector disassembly, limiting their clinical applications. [10–13] Alternatively, polymeric NPs that contain nucleic acids can be composed of solid polymer matrix such as poly(lactide-co-glycolide) (PLGA) with the polycation encapsulated or attached to the particle surface.[14] Nanoparticles composed of PLGA are attractive for silencing applications because of their high stability, low toxicity and the possibility of controlled release of the cargo, unfortunately the knockdown efficiency is not as high as that observed with the polyplex systems.[15] To date, there have been no reports of intravenously delivered PLGA based carriers and there are several reports of the *in vitro* delivery of siRNA by PLGA.[16,17] On the other hand, liposomes have proven to be highly efficient carriers of siRNA, especially to hepatocytes.[18] Neutral lipids exhibit low toxicity, low immunogenicity and are easily produced. Liposomes are also an attractive choice for gene delivery because they can be formulated as ~100 nm in size and their by-products are biocompatible. Lipid encapsulation of the siRNA simply involves mixing and incubation with a mixture of cationic and neutral lipid.[19] Specific delivery can be achieved by conjugating a targeting ligand to the lipid molecule and then processing to form liposomes. The cationic charge can electrostatically complex with siRNA to achieve a more robust construct, while the neutral lipids facilitate fusion to the host cell's membrane.[20,21] Generally both liposomes and polymeric nanoparticles however are formed

by self-assembly and so are not structurally robust. This represents a major disadvantage from the standpoint of reproducibility, manufacturing, and drug administration.[22]

Hybrid NPs, formed by combining polymers and lipids, can be a robust drug delivery platform with high drug encapsulation yields, tunable and sustained drug release profiles, and excellent serum stabilities.[23, 24] Polymeric NPs have been mixed with liposomes to form lipid-polymer complexes such as lipoparticles where the lipid bilayer or lipid multilayer fuses on the surface of polymeric NPs.[25] These complexes usually require a two-step formulation process: 1) development of polymeric NPs, and 2) encapsulation of polymeric NPs within liposomes. Additionally, a platform to engineer sub-100 nm targeted lipid-polymer hybrid NPs through a combination of nanoprecipitation and self-assembly has been developed.[26]

We are interested in developing a process that enables the formation of a simple two-component PLGA-siRNA nanoparticle system where the particle formation step does not require a polyplex. Such a system, intrinsically resulting in particles with a negative zeta potential with the siRNA protected as a cargo, could have its surface charge adjusted with the addition of cationic lipids or polymers. Specifically, we have used a top-down fabrication method, PRINT®, to generate highly uniform nanoparticles with a polymeric core encapsulating the siRNA and a lipid shell enabling cellular uptake of the nanoparticle formulation.[27] We have shown that polylactic-co-glycolic acid (PLGA) particles containing siRNA made with this approach can efficiently cause knockdown of gene expression in various prostate cancer cell lines with minimum cytotoxicity.

The PRINT technique was used as described previously with a slight modification to fabricate 80×320 nm monodisperse, siRNA-encapsulated PLGA particles followed by coating with a cationic lipid.[27] Non-spheical nanoparticles were specifically chosen for these studies because needle-shaped polymer nanoparticles have been shown to be internalized more readily than their spherical counterparts, causing a greater percentage of gene knockdown in endothelial cells.[28] Briefly, a pre-particle solution containing PLGA (85:15 lactic acid: glycolic acid, MW=55,000g/mol, purchased from Sigma Aldrich) with siRNA (purchased from Dharmacon) was prepared in a DMSO:DMF:water solvent mixture (4:16:1) and cast on a poly(ethylene teraphthalate) (PET) sheet (delivery sheet). The delivery sheet was then placed in conformal contact with a PRINT mold with 80×320 nm features patterned. The mold was kindly provided by from Liquidia Technologies (Product # MCI-300-162B, 6” wide). The delivery sheet and mold were passed through a heated laminator (150°C, 5.5 $\times 10^5$ Pa) and separated at the nip. This process enables the siRNA/polymer to fill the molds thereby forming nanoparticles. To harvest the nanoparticles from the mold, the mold was placed in conformal contact with a PET sheet that was coated with a layer (400 nm cast from water) of poly(vinyl alcohol) (PVA, MW=2000g/mol). The mold/PET-PVA ensemble was again passed through the laminator (150°C, 5.5 $\times 10^5$ Pa). Both the filling of the mold and the transferring of the particles onto the PVA coated PET sheet were performed at low humidity (~20–30%). The particles were then released from the PET/PVA sheet by using a bead of water (~1mL) to dissolve the PVA layer thereby removing the particles from the PET sheet. This process was accomplished using a simple bead harvester. A 1 mg/mL stock solution of DOTAP:DOPE (1:1 wt%, purchased from Avanti Polar Lipids) in water was used to release the particles from the PET sheet by dissolving the PVA coating. Six feet of particles were harvested in 1 mL of 1 mg/mL lipid (1 mg of lipid for 6 ft of particles – 0.17 mg lipid/ft of particles). The typical yield of particles was 0.4 mg particles/ft of PRINT mold. Therefore 0.4 mg of particles was coated with 0.17 mg of lipid, resulting in a particle to lipid mass ratio 1:2.4. To remove excess lipid and PVA and to concentrate the particles, tangential flow filtration (TFF) was used to concentrate the particle dispersion to a concentration of 2 mg/mL. The particles were then lyophilized by adding 10×

mannitol and 8× sucrose (10× and 8× to mass of particles) using a tree lyophilizer. Mannitol and sucrose were used as cryoprotectants. The ratio of particles produced to harvesting lipid determines the zeta potential of the particles (Figure 1).

The particles were characterized using dynamic light scattering (DLS) (size and zeta potential measurements) and both scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Figure 2 and Table 1). PLGA particles that are harvested in pure water were negatively charged (−4.9 mV), whereas the lipid-coated particles are positively charged and the charge could be controlled over a wide range (0–20 mV). The stability of the particles in PBS and serum was investigated at physiological conditions. As seen in Figure S1, the size of the particles was found to be stable up to one week at at 37 °C in phosphate buffer and 10% serum in phosphate buffer. PLGA is a biodegradable polymer and so with time the polymer degrades into lactic acid and glycolic acid in water. Interestingly, the zeta potential of the lipid-coated PLGA/siRNA particles was negative when the particles were placed in 10% serum, suggesting the adsorption of serum proteins. This result is not surprising because serum is composed of negatively charged proteins. The release profile of the siRNA from the PLGA in PBS at 37 °C was determined by extracting the siRNA from the remaining particle solution and running a 2.5% agarose gel (Figure S2). In addition, the stability of the siRNA in the lipid-coated PLGA/siRNA particles in cell culture media was evaluated by incubating the particles in cell culture media for 0, 0.5, 1, 4, and 24 h at 37°C and extracting the siRNA and running a 2.5% agarose gel (Figure S3). The siRNA bands in the agarose gel indicate that the siRNA is stable in the lipid-coated PLGA/siRNA particles upto 24 h in cell culture media.

siRNA loading capacity of particles was examined by dissolving the particles in chloroform and extracting siRNA with 0.1% SDS/0.5 M NaCl aqueous solution. When loaded on 2.5% agarose gel, intact particles did not go into 2.5% agarose gel due to their size (Figure 3). siRNA extracted with the above method formed sharp bands in gel, and was imaged and quantified to be between 1–2 wt% siRNA in the particles. Thus the encapsulation efficiency of this particle fabrication approach is between 20–40 % (1–2 wt% vs. 5 wt% charged), which is in the range of encapsulation efficiencies for PLGA formulations (15–43%).[29] By using a PLGA with a lower molecular weight and different lactic acid to glycolic acid ratio we were able to increase the encapsulation of siRNA (Table 2).

Cationic particles were found to be readily internalized into cells.[30] Dye-labeled siRNA (Alexafluorophore AF547) was used to evaluate the internalization of lipid-coated PLGA/siRNA nanoparticles by various cell lines including: Raw264.7 (mouse macrophage), HeLa/luc (human cervical cancer), PC3, DU145, LNCaP (all human prostate cancer), and HepG2 (human liver carcinoma). Figure 4 illustrates that these different types of cell lines were all able to internalize the lipid-coated PLGA/siRNA particles. While HeLa/luc, PC3, DU145 and Raw264.7 cells showed very high uptake of particles, i.e.~100% of the cells were particle-positive after 4 h dosing with 100 ug/mL of particles, LNCaP and HepG2 cells were not very efficient in taking up particles because their doubling time is 60 h and 48 h respectively which is significantly longer than the other cell lines (Table S1). The variation in the kinetics of cellular uptake for the same type of nanoparticle on different cell lines has been reported. Internalization of particles by multiple cell lines was further confirmed by confocal microscopy (Figure 5). To confirm that the images in Figure 5 were not simply of adsorbed versus internalized particles, a sample Z-stack image of the HeLa cells dosed with 100 μg/mL of lipid-coated PLGA/siRNA particles is included in Figure S4 which confirms the particle internalization.

Luciferase-expressing HeLa (HeLa/luc) cells were used to test the efficacy of lipid-coated PLGA/siRNA nanoparticle formulation to silence gene expression. HeLa/luc cells were

dosed for 4 h with lipid-coated PLGA/siRNA particles (5 wt% luciferase or irrelevant control siRNA and 95 wt% PLGA, coated with 0.17 mg DOTAP:DOPE per ft of PRINT mold corresponding to 1:2 lipid:particle by mass) and incubated for 72 h. The cells incubated with the particles containing the luciferase siRNA resulted a decrease in the luciferase expression in comparison to the untreated cells, whereas the cells treated with the particles that were loaded with the control sequence did not exhibit a decrease in the luciferase expression. The half maximal inhibitory concentration (IC_{50}) value of these PRINT particle formulation (47 nM) was comparable to that of Lipofectamine 2000 transfection (55 nM) (Figure 6). Knockdown of luciferase expression can be achieved at 24 h and 48 h of incubation as well (Figure S5). The IC_{50} value of the particles could be varied by varying the zeta potential of the particles (which can be varied by changing the lipid: particle ratio) (Figure 7)). The higher the zeta potential, the lower IC_{50} was obtained. However, cytotoxicity of particles also increased with zeta potential. We chose 0.17 mg/mL (~+3 mV) as the appropriate lipid solution concentration because it resulted in a relatively low IC_{50} value (47 nM) as well as minimal cytotoxicity at the highest concentration of particles tested (200 ug/mL).

After demonstrating that the lipid-coated PLGA/siRNA particles were able to deliver luciferase siRNA and knockdown luciferase expression, we further evaluated our formulation with a therapeutically relevant siRNA target. KIF11 (also known as Eg5, purchased from Dharmacon) encodes a motor protein that belongs to the kinesin-like protein family that is involved in bipolar spindle formation during cell mitosis. KIF11 represents an attractive anti-cancer target, and inhition of KIF11 is known to cause mitotic arrest and apoptosis of multiple cancers.[31–34] Three prostate cancer cell lines (LNCaP, PC3, and DU145) were dosed with 100 μg/mL of lipid-coated PLGA particles carrying KIF11 siRNA or irrelevant control siRNA for 4 h, followed by 72 h incubation in complete growth medium. Quantitative RT-PCR results showed a statistically significant decrease in KIF11 mRNA level in all three prostate cancer cells lines dosed with KIF11 siRNA PLGA particles while control siRNA-loaded particles did not show a decrease in KIF11 mRNA level (Figure 8A). The statistical analysis was done by comparing the knockdown of lipid-coated PLGA/siRNA particles containing KIF11 siRNA and control siRNA using the Wilcoxon Rank test. For all the three different cell lines the p value was < 0.01. Delivery of KIF11 siRNA by the lipid-coated PLGA carrier also induced a significant decrease in the cell viability of the three cell lines as shown by a CellTiter-Glo viability assay (Figure 8B). To confirm the down regulation of KIF11 protein, Western Blot analysis was also performed (Figure S6). Currently we are working on the *in vivo* delivery of siRNA for prostate cancer and other targets.

Herein we have developed a process that enables the formation of a simple two-component PLGA-siRNA nanoparticle system. The formation of a polyplex has been avoided using the PRINT process and transfection achieved via surface modification of particles with lipids. The internalization of PRINT lipid-coated PLGA/siRNA nanoparticles by multiple cancer cell lines and the knockdown therapeutically relevant genes has been demonstrated. We are now investigating the *in vivo* efficacy of lipid-coated PLGA-siRNA nanoparticles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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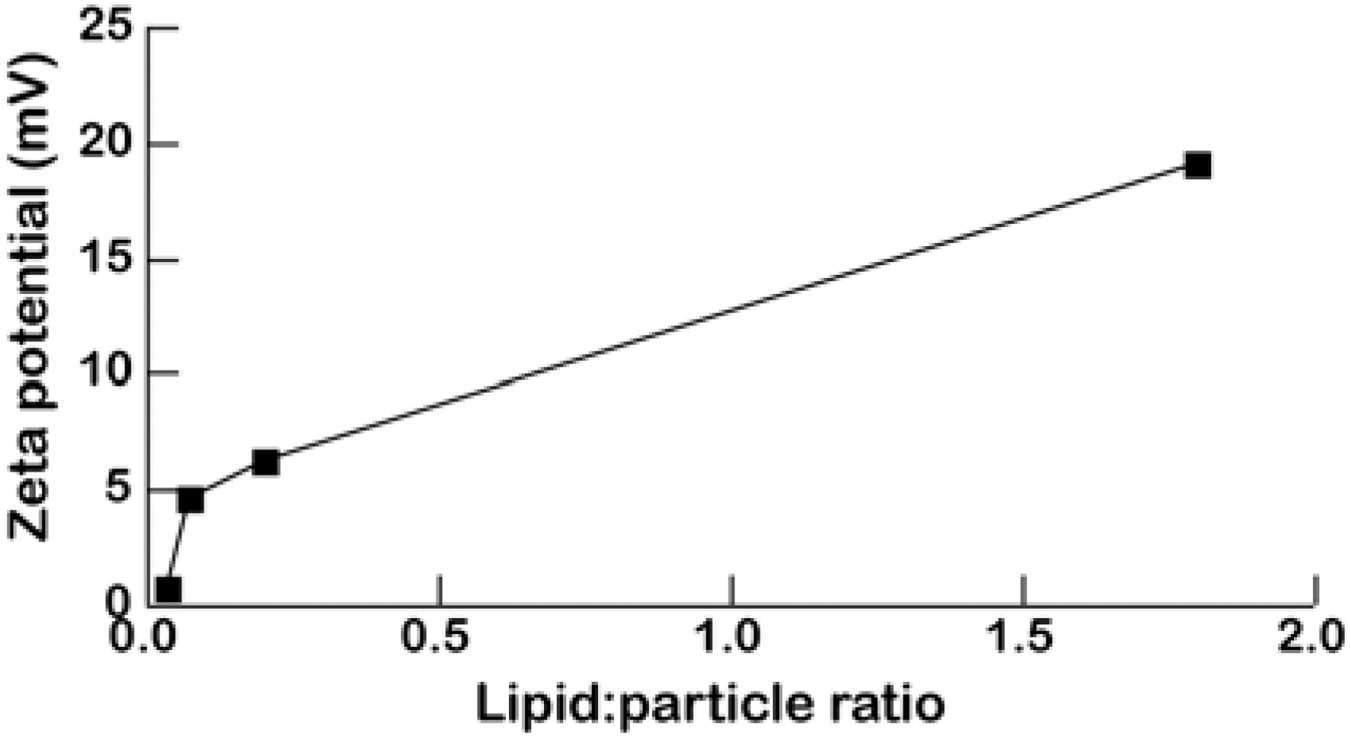

Figure 1.
The zeta potential of the particles can be varied by changing the lipid:particle ratio based on the harvesting lipid concentration to particle concentration.

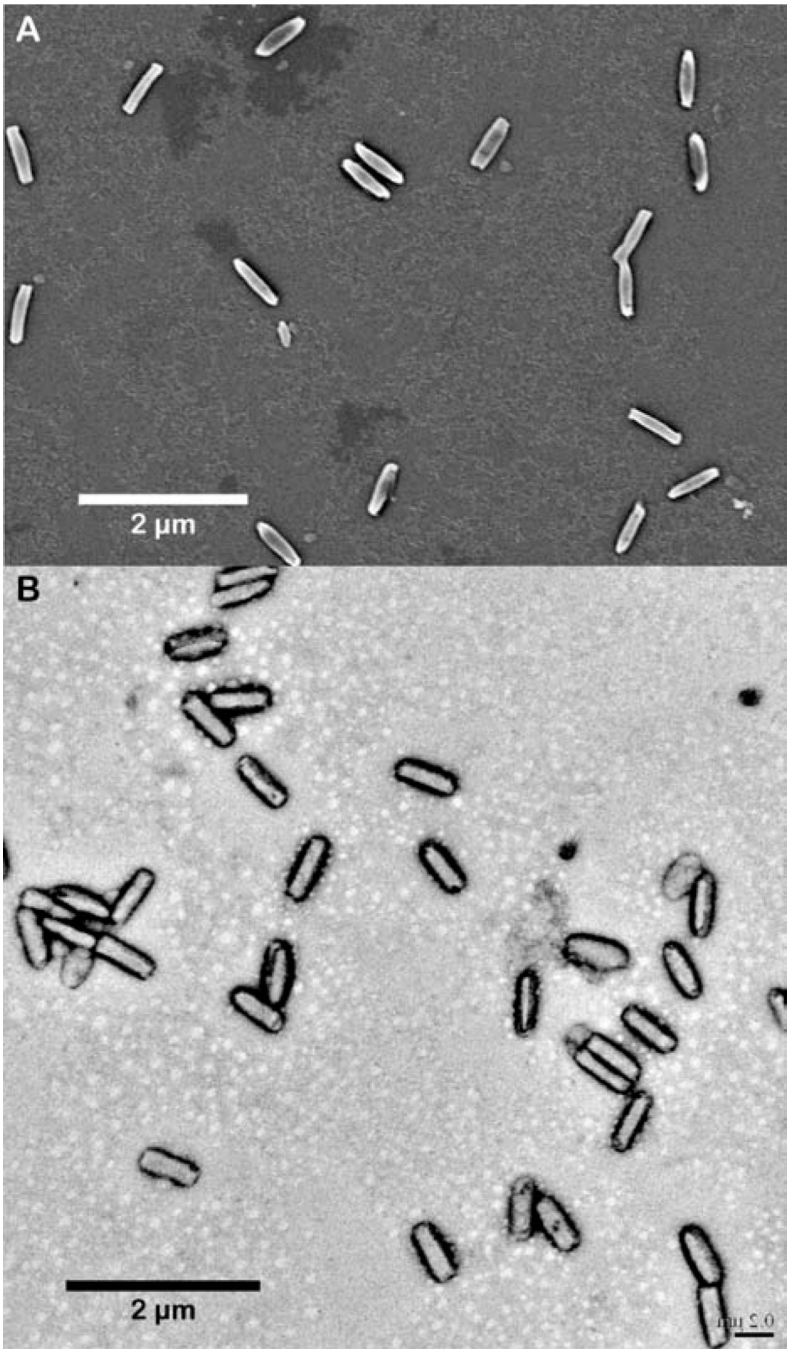

Figure 2.
A) SEM image of lipid-coated PLGA/siRNA nanoparticles. B) TEM image of lipid-coated PLGA/siRNA nanoparticles.

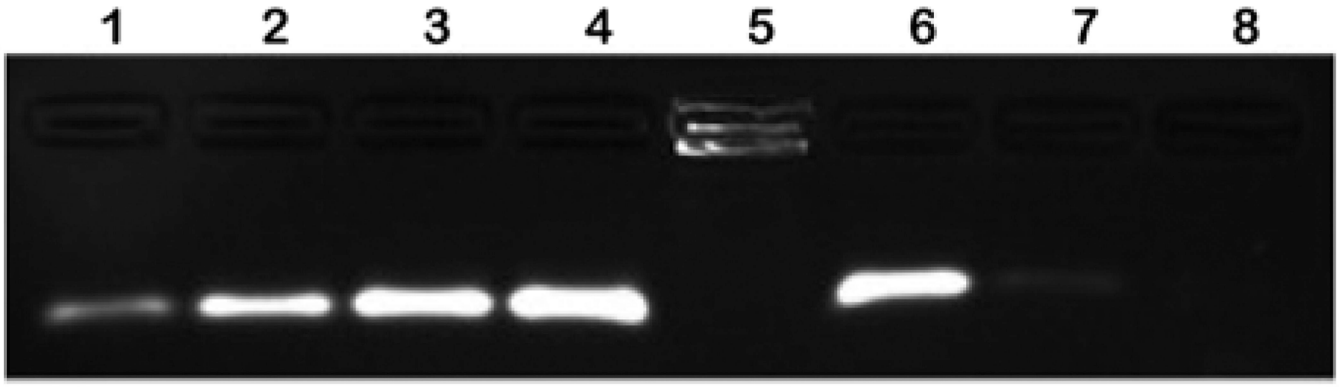

Figure 3.
Determination of siRNA encapsulation. 1, 50 ng siRNA; 2, 100 ng siRNA; 3, 150 ng siRNA; 4, 200 ng siRNA; 5, 20 μg lipid-coated PLGA/siRNA nanoparticles; 6–8, three successive siRNA extractions from lipid-coated PLGA/siRNA nanoparticles with chloroform/0.1% SDS-0.5M NaCl, equivalent to 20 μg particles. Samples were run on 2.5% agarose gel and imaged with ImageQuant LAS4000 (GE Healthcare).

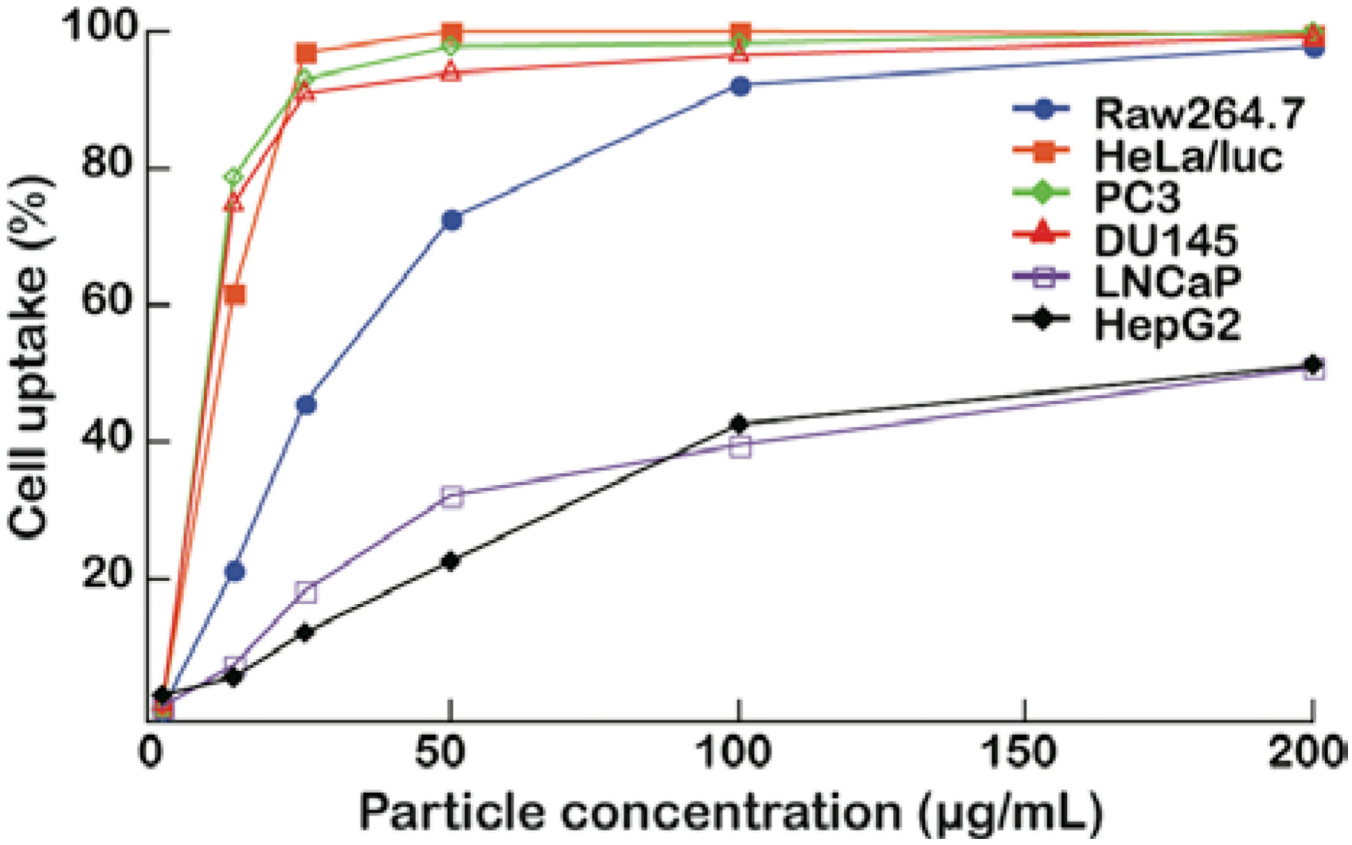

Figure 4.

Cellular internalization of lipid-coated particles. Cells were dosed with 80×320 nm particles loaded with Alexa Fluor 547 labeled siRNA at 50 μg/mL for 4 h. Then particles were removed and cells were washed 3 times with PBS. Trypsinized cells were analyzed by a Cyan ADP flow cytometer (Dako). Cell uptake was expressed as percentage of cells that were fluorescence positive.

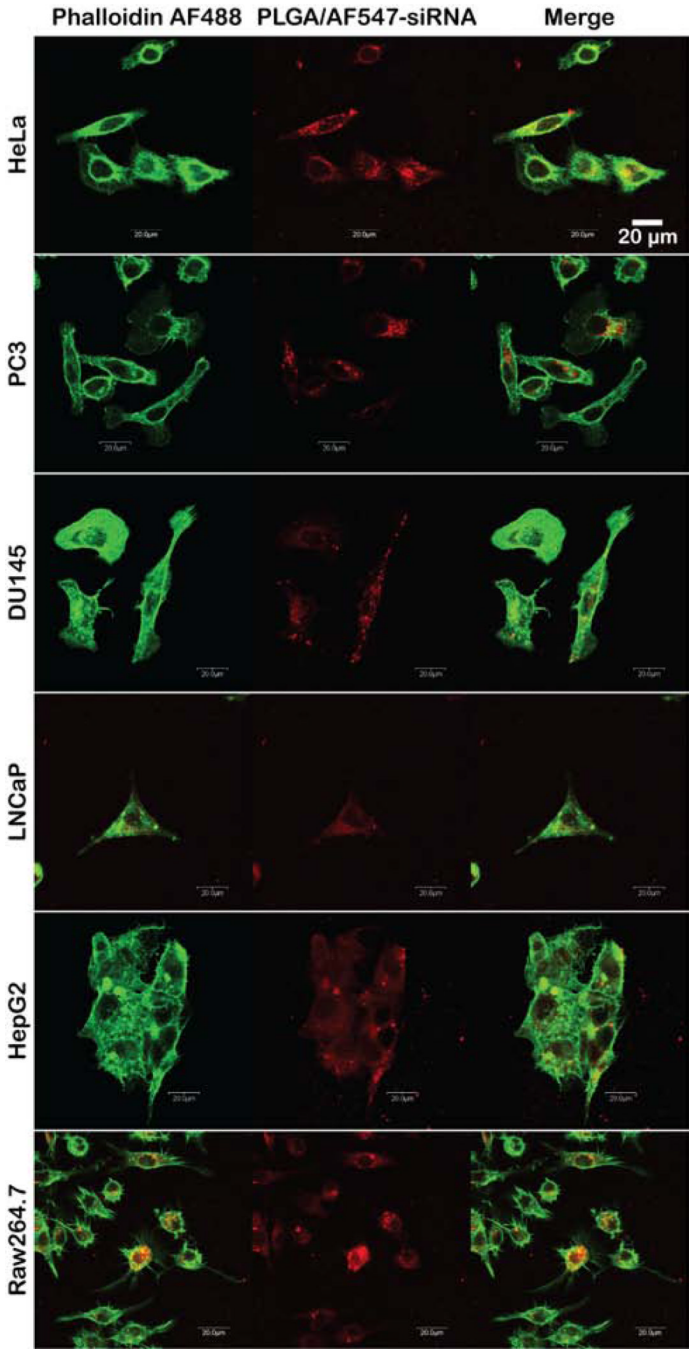

Figure 5.

Cellular internalization of lipid-coated PLGA/siRNA particles by confocal microscopy. Cells were treated with 100 μg/mL particles loaded with Alexa Fluor 547 conjugated siRNA for 4 h at 37°C. Cells were then fixed and stained with Alexa Fluor 488 labelled phalloidin. All the images are on the same scale. The brightness of the confocal images was enhanced.

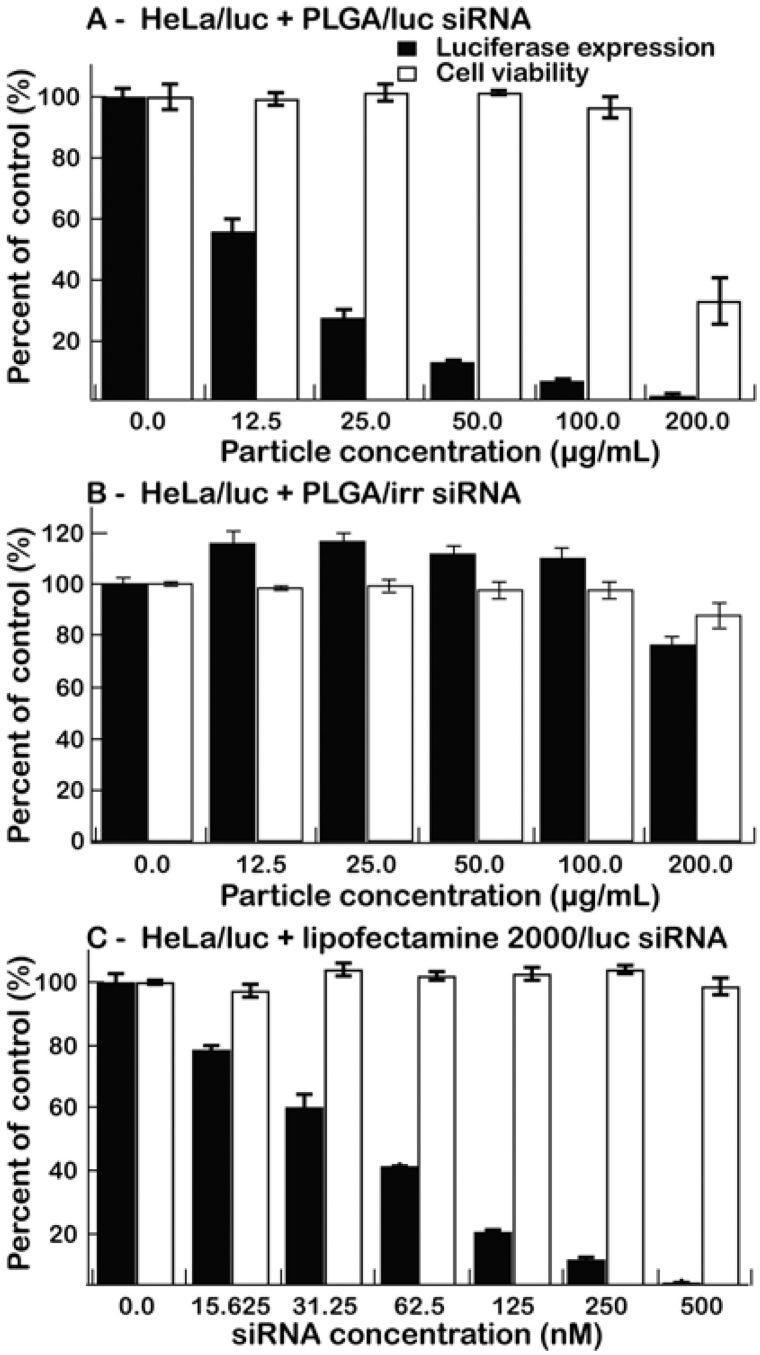

Figure 6.

Knockdown of luciferase gene expression in HeLa/luc cells using lipid-coated PLGA/siRNA nanoparticles with (A) antiluciferase siRNA, (B) control siRNA, and (C) transfection of luciferase siRNA with Lipofectamine 2000.

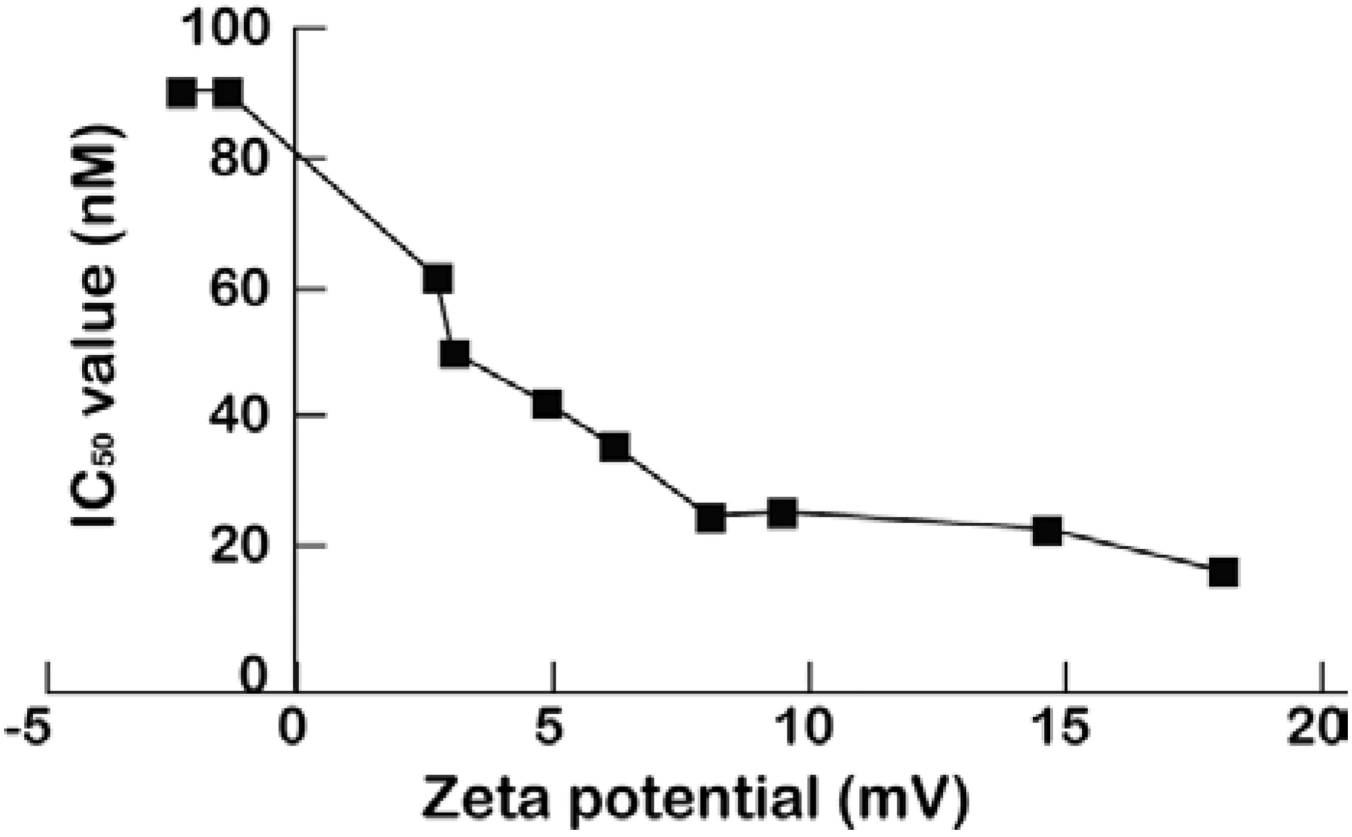

Figure 7.
IC_{50} of luciferase gene knockdown on HeLa/luc cells by lipid-coated PLGA particles can be tuned by changing the zeta potential of nanoparticles.

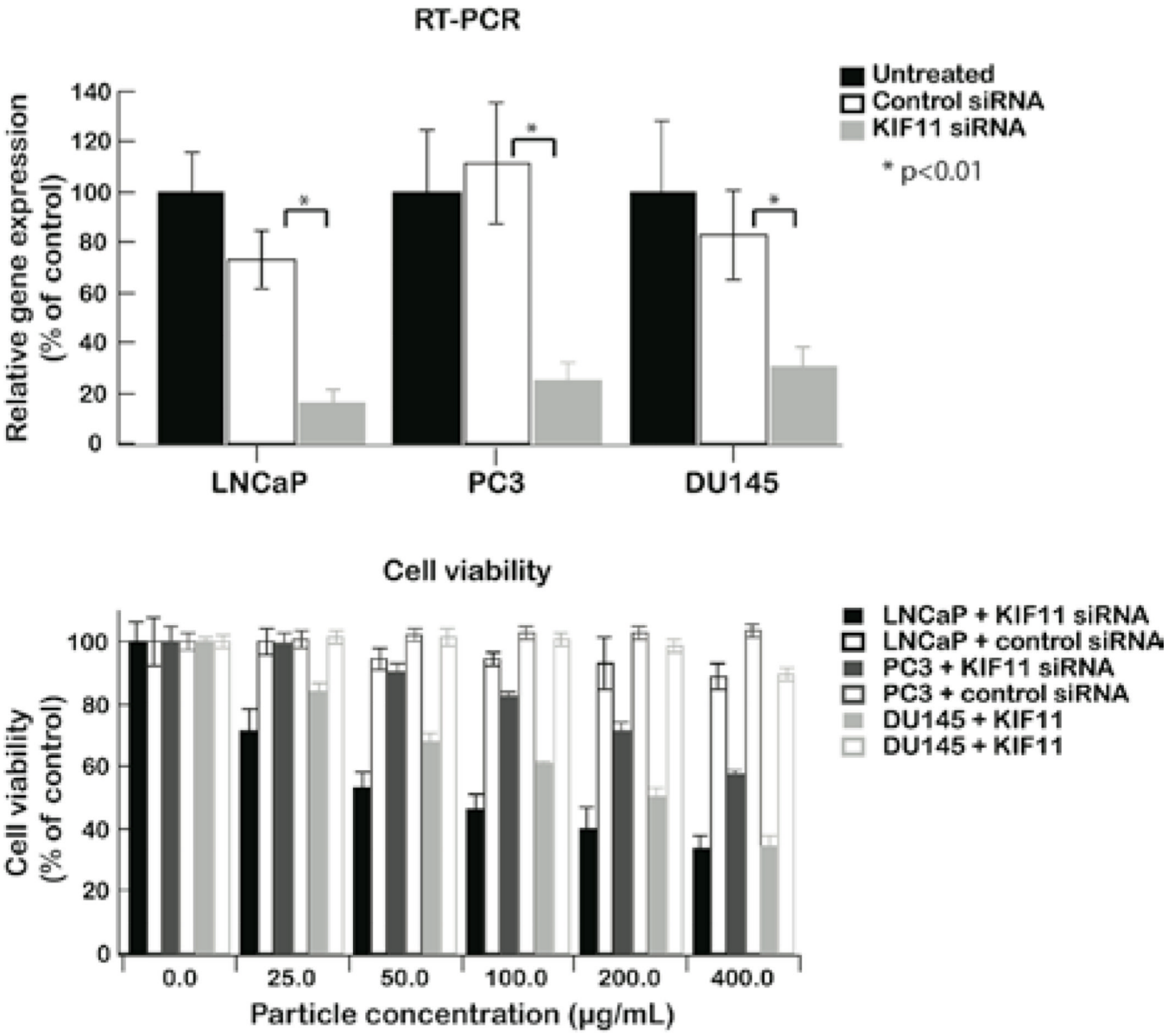

Figure 8.

Delivery of therapeutic siRNA to prostate cancer cells. Cells were dosed with particles for 4 h at 37°C, followed by 72 h incubation in complete growth medium. qRT-PCR (top) and CellTiter-Glo viability assay (promega) (bottom).

Table 1

Particle characterization by dynamic light scattering.

Particle	Size (nm)	PDI	Zeta potential (mV)
PLGA-siRNA	198 ± 3.45	0.045 ± 0.009	−3.45 ± 1.9
PLGA-siRNA-lipid (0.2 mg lipid/ft particles)	207 ± 4.461	0.092 ± 0.005	5.29 ± 1.5

Table 2

Encapsulation efficiency of siRNA in different types of PLGA.

Polymer	Total siRNA (wt%)	Encapsulation efficiency (%)
33K PLGA (50:50)	2.3	46
55K PLGA (85:15)	1.61	32