Video Article

Porous Silicon Microparticles for Delivery of siRNA Therapeutics

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Abstract

Small interfering RNA (siRNA) can be used to suppress gene expression, thereby providing a new avenue for the treatment of various diseases. However, the successful implementation of siRNA therapy requires the use of delivery platforms that can overcome the major challenges of siRNA delivery, such as enzymatic degradation, low intracellular uptake and lysosomal entrapment. Here, a protocol for the preparation and use of a biocompatible and effective siRNA delivery system is presented. This platform consists of polyethylenimine (PEI) and arginine (Arg)-grafted porous silicon microparticles, which can be loaded with siRNA by performing a simple mixing step. The silicon particles are gradually degraded over time, thereby triggering the formation of Arg-PEI/siRNA nanoparticles. This delivery vehicle provides a means for protecting and internalizing siRNA, without causing cytotoxicity. The major steps of polycation functionalization, particle characterization, and siRNA loading are outlined in detail. In addition, the procedures for determining particle uptake, cytotoxicity, and transfection efficacy are also described.

Video Link

The video component of this article can be found at https://www.jove.com/video/52075/

Introduction

Small interfering RNAs (siRNAs) are double-stranded RNA molecules that can suppress the expression of genes. In recent years, siRNAs have been developed as a new generation of biodrugs that show therapeutic potential for future use in clinical applications[1-5]. However, the successful implementation of siRNA therapy remains a considerable challenge, due to degradation by nucleases, poor intracellular uptake, low transfection efficiency and inefficient release from the endosome/lysosome[5]. Many of these hurdles can be overcome by the development of delivery platforms, which can safely and efficiently deliver siRNA to diseased tissue. Compared to viral carriers, non-viral platforms provide several advantages, such as safety, low cost and ease of tailoring. In particular, cationic nanoparticles, such as polymers and lipids, have proved useful for siRNA delivery[3].

Previously, we have developed a discoidal drug delivery system, termed the multistage vector (MSV). This platform is based on sequential stages, in which one vehicle is released from another. The first stage vehicle is a microparticle made from biodegradable porous silicon (pSi), while the second stage vehicles are nanoparticles loaded with drugs or contrast agents[6,7]. The nanoparticles, which are embedded in the pSi material, are gradually released as the Si degrades[8]. A benefit of using Si particles is that the morphology and surface characteristics can easily be tailored to achieve optimal biodistribution and drug release. Recently, the successful use of the MSV platform for the delivery of siRNA liposomes to tumor tissue was shown in an ovarian and breast cancer mouse model[9, 10].

In this work, we have fabricated a universal delivery system for siRNA based on the principals of the MSV platform. The efficacy of this delivery system has previously been demonstrated using different siRNA molecules[11]. The system is a polycation-functionalized porous silicon (PCPS) carrier, consisting of pSi grafted with polyethylenimine (PEI) and arginine (Arg). PEI can aid in forming electrostatic interactions with siRNA, while Arg and pSi can serve to reduce the toxicity of PEI, as previously demonstarted[11]. In addition, the presence of PEI can assist in intracellular uptake and endosomal escape, while the pSi microparticles enable siRNA protection and sustained release. The pSi particles gradually degrade under physiological conditions, thereby resulting in the formation of Arg-PEI/siRNA nanoparticles (**Figure 1**), which have a distinct morphology and a narrow size distribution[11]. For details regarding the stability of the PCPS/siRNA system, please refer to the study by Shen *et al.*[11]. This PCPS platform differs from the conventional MSV, since the second stage nanoparticles are not initially present in the carrier, but are formed over time as the first-stage carrier degrades[11, 12]. The siRNA loading efficiency, cytotoxicity and gene silencing efficiency of the PCPS system

has been evaluated *in vitro*. Transfection efficiency was measured using siRNA against the ataxia telangiectasia mutated (ATM) oncogene, which is involved in DNA repair[10]. Previously, the suppression of ATM has been shown to decrease tumor growth in a breast cancer model[10].

Protocol

1. PCPS Particle Preparation

1. Oxidize non-functionalized porous silicon particles in a 30% solution of hydrogen peroxide at 95 °C for 2 hr. Aminate the oxidized particles in 2% (3-aminopropyl)triethoxysilane solution in isopropyl alcohol for 2 days at 65 °C with gentle stirring.
2. Centrifuge the solution for 30 min at 18,800 x g and wash the particles twice in isopropyl alcohol and three times in ethanol, using brief sonication to suspend the pellet. Leave the particles in ethanol solution when performing step 1.3 and 1.4.
3. Add a known volume of particle suspension (*e.g.*, 10 µl) to 10 ml isotone diluent and count the particles with a particle counting analyzer to determine the concentration of particles in the stock solution.
4. Activate the acid group of L-arginine (0.1 nmol) with *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC, 0.1 nmol)/*N*-hydroxysuccinimide (NHS, 0.1 nmol) in 20 ml of ethanol for 2 hr at RT with gentle stirring.
5. Briefly sonicate the particle stock solution and add 1 billion particles to the L-arginine solution and leave the reaction for 18 hr at RT with gentle stirring.
6. Activate the first aspartic acid group of *N*-(tert-Butoxycarbonyl)-L-aspartic acid (Boc-Asp-OH, 1 nmol) with EDC (0.1 nmol)/NHS (0.1 nmol) in 20 ml of ethanol for 4 hr at 4 °C with gentle stirring.
7. Dissolve 50 mg polyethylenimine in 10 ml ethanol and add the solution to the Boc-Asp-OH mixture. Let the reaction proceed for 24 hr at RT with gentle stirring.
8. Activate the second aspartic acid group of the Boc-Asp-OH/PEI solution with EDC (0.1 nmol)/NHS (0.1 nmol) at 4 °C for 6 hr with gentle stirring.
9. To obtain PCPS particles, add the particle solution from step 1.5 into the Boc-Asp-OH/PEI solution from step 1.8. Allow the reaction to proceed for 18 hr at RT with gentle stirring.
10. Centrifuge the solution for 30 min at 18,800 x g and wash the particle solution three times with ethanol, using brief sonication to suspend the pellet.

2. PCPS Particle Characterization

1. Measure the size of the particles using a scanning electron microscope (SEM).
    1. Place a drop of particle suspension (10,000 particles/µl in ethanol) on a clean silica SEM sample stub and let dry at RT under vacuum.
    2. Measure SEM images at 8 kV with a 3–5 mm working distance using an in-lens detector.

2. Measure the zeta potential of the particles using a particle analyzer system.
    1. Mix 10 µl of particle suspension (10,000 particles/µl in ethanol) with 1 ml of 10 mM phosphate buffer (pH 7.4).
    2. Load the sample into folded capillary cells and measure the zeta potential according to the manufacturer's instructions.

3. Loading of siRNA into PCPS Particles

1. Dry the PCPS particles (from PCPS particle preparation step 1.9) under vacuum O/N.
2. Add siRNA (4 µg) in nuclease-free water (20 µl) to the dried PCPS particles and sonicate briefly. Use the following particle to siRNA ratios: 2×10^5 particles/0.2 µg siRNA, 4×10^5 particles/0.2 µg siRNA, 6×10^5 particles/0.2 µg siRNA, 8×10^5 particles/0.2 µg siRNA, 10×10^5 particles/0.2 µg siRNA and 12×10^5 particles/0.2 µg siRNA.
3. Incubate for 3 hr at 4 °C on a shaker (1,000 rpm) to allow siRNA binding to the particles.

4. Optimization of siRNA/PCPS Particle Ratio

1. Add DNA loading dye to 20 µl of the PCPS/control siRNA particles with different particle to siRNA ratios (see loading of siRNA into PCPS particles).
2. Load the samples into a 2% agarose gel containing DNA gel stain.
3. Perform electrophoresis at a constant voltage of 120 V for 20 min in running buffer.
4. Analyze the gel with image acquisition and analysis software.

5. Release of siRNA from PCPS Particles

1. Mix 20 µl of the PCPS/control siRNA particles with different particle to siRNA ratios (see step 3) in sodium dodecyl sulfate (SDS, 2%) and let stand for 1 hr at RT.
2. Add DNA loading dye to the samples.
3. Load samples into a 2% agarose gel containing DNA gel stain.
4. Perform electrophoresis at a constant voltage of 120 V for 20 min in running buffer using DNA electrophoresis equipment and a power supply.
5. Analyze the gel with image acquisition and analysis software.

6. Confocal Microscopy of PCPS Particles

1. Add 5 µl of PCPS/fluorescent control siRNA particles (10×10^5 particles/0.2 µg siRNA/20 µl) to a glass cover slip.
2. Visualize particle layers by confocal microscopy.

7. Characterization of Arg-PEI/control siRNA Nanoparticles

1. To degrade the silicon material and form Arg-PEI/control siRNA nanoparticles, add PCPS/siRNA particles (10×10^6 PCPS particles/2 µg siRNA) to 100 µl of phosphate buffered saline and shake (1,000 rpm) at 37 °C for 2 days.
2. Centrifuge the sample for 30 min at 18,800 x g and collect the supernatant.
3. Measure the size of the formed Arg-PEI/siRNA nanoparticles with dynamic light scattering (DLS).
    1. Mix 10 µl of the supernatant with 1 ml 10 mM phosphate buffer (pH 7.4) and place into a plastic cuvette.
    2. Measure the size of the particles using a particle analyzer system according to the manufacturer's instructions.
4. Determine the size and morphology of the formed Arg/PEI-siRNA nanoparticles with atomic force microscopy.
    1. Take 10 µl of the supernatant and place on a silicon wafer.
    2. Visualize the particles with atomic force microscopy (AFM).

8. Cell Culture

1. Culture MDA-MB-231 human breast cancer cells in cell culture media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 5% CO_2, 95% humidity and 37 °C.

9. Confocal Microscopy of Live Cells with PCPS Particles

1. Plate cells in 2-well culture slides at a seeding density of 3×10^5 cells/well for 24 hr.
2. Add PCPS particles loaded with fluorescent control siRNA (10×10^5 particles/0.2 µg particle to siRNA ratio, 50 nM siRNA) to cells.
3. Record a movie of the cells with a confocal microscope (supplied with a chamber, 5% CO_2, 95% humidity and 37 °C) for 12 hr following particle exposure.

10. Confocal Microscopy of Fixed Cells with PCPS Particles

1. Plate cells in 2-well culture slides at a seeding density of 3×10^5 cells/well for 24 hr.
2. Add PCPS particles loaded with fluorescent control siRNA (10×10^5 particles/0.2 µg particle to siRNA ratio, 50 nM siRNA) to cells and incubate for 1 day, 7 days and 10 days.
3. Wash the cells twice with phosphate buffered saline and then fix them with 4% paraformaldehyde solution for 10 min.
4. Wash the cells with phosphate buffered saline.
5. Permeabilize the cells with 0.1% octyl phenol ethoxylate for 10 min and then wash them three times with phosphate buffered saline.
6. Block the cells with albumin from bovine serum (10 mg/ml) in phosphate buffered saline for 10 min at RT with gentle stirring.
7. To visualize filamentous actin, incubate the cells with fluorescently labeled phalloidin (1 µl/40 µl blocking solution) for 20 min at RT with gentle stirring and then wash in phosphate buffered saline.
8. Remove the slides from the frame and add antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) to visualize the nucleus.
9. Add a cover glass on top and take images of the cells with confocal microscopy.

11. Flow Cytometry of Cells with PCPS/fluorescent Control siRNA Particles

1. Plate cells in a 6-well plate at a seeding density of 3×10^5 cells/well for 24 hr.
2. Add PCPS particles loaded with fluorescent control siRNA (10×10^5 particles/0.2 µg particle to siRNA ratio, 50 nM siRNA) to the cells and incubate for 24 hr.
3. Wash the cells with phosphate buffered saline and scrape them with a cell scraper.
4. Store the cells in phosphate buffered saline with 2% fetal bovine serum prior to analysis. Use untreated cells as a negative control.
5. Perform flow cytometry.

12. Cell Viability of Cells with PCPS Particles and PCPS/control siRNA Particles

1. Plate cells in a 96-well plate at a cell density of 3×10^3 cells/well for 24 hr.
2. Treat the cells with PCPS particles (1.5×10^5/well and 6×10^5/well) or PCPS/control siRNA particles (10×10^5 particles/0.2 µg particle to siRNA ratio, 10 nM and 100 nM siRNA) for 48 hr and 72 hr. Use untreated cells and cells treated with phosphate buffered saline (same volume as the added particles) as controls. Assay each sample in triplicate.
3. Perform a cell proliferation assay according to the manufacturer's instructions.
4. Represent data as the mean ± standard deviation.

13. Western Blot of Cells with PCPS/ATM Mutated siRNA Particles

1. Plate cells in a 6-well plate at a cell density 2 × 10^5 cells/well for 24 hr.
2. Incubate the cells with PCPS/control siRNA (50 nM) particles or PCPS/ATM siRNA (50 nM) particles for 72 hr. Use untreated cells as a control.
3. Lyse the cells using a protein extraction reagent supplemented with a protease inhibitor cocktail.
4. Centrifuge the cell lysates for 10 min at 14,000 x g and recover the supernatant.
5. Determine the protein concentration with a protein quantification assay according to the manufacturer's instructions.
6. Add sample loading buffer (with 5 µl 2-mercaptoethanol/ml buffer) to the samples and heat them for 6 min at 99 °C.
7. Load the protein samples (20 µg/µl) in a 12% SDS-polyacrylamide gel in running buffer and perform polyacrylamide gel electrophoresis (1 hr, 120 V) using electrophoresis equipment and a power supply.
8. Transfer the gel in transfer buffer (with 20% methanol) to a nitrocellulose membrane (1 hr, 100 V) using electrophoresis equipment and a power supply.
9. Block the membrane with 5% dry milk for 1 hr.
10. Incubate the membrane with the ATM primary antibody (from rabbit) in blocking solution (from step 13.9) at a 1:1,000 dilution O/N.
11. Wash the membrane with phosphate buffered saline containing 0.1% polyethylene glycol sorbitan monolaurate and then incubate it with the secondary antibody (anti-rabbit) in blocking solution (from step 13.9) at a 1:2,500 dilution for 1 hr.
12. Wash the membrane with phosphate buffered saline containing 0.1% polyethylene glycol sorbitan monolaurate and detect the protein bands with Western blot detection reagent using image acquisition and analysis software.
13. For the loading control, wash the membrane and repeat steps 13.9-13.12 using a β-actin primary antibody (from mouse, 1:10,000 dilution) and a secondary antibody (anti-mouse 1:4,000 dilution).

Representative Results

This protocol describes the use of a non-viral delivery system for safe and efficient siRNA transfection. The SEM results reveal that the PCPS particles are cylindrical in shape and have a diameter of 2.6 µm (**Figure 2A**). The particles are positively charged with a zeta potential of approximately +8.21 (**Figure 2B**), thereby enabling electrostatic binding with negatively charged nucleotides. Confocal images of different layers of the PCPS particles demonstrate that fluorescent control siRNA is loaded inside the porous silicon particles (**Figure 2C**). The formation and release of Arg-PEI/siRNA nanoparticles from the pSi particles was confirmed with DLS and AFM. The size distribution of the particles ranged from 70-120 nm, with an average size of 94 nm (**Figure 2D**). AFM images illustrate that the nanoparticles have a spherical shape (**Figure 2E**).

The ratio of particles to siRNA was optimized by agarose gel electrophoresis to ensure high binding affinity (**Figure 3A**)**.** A wide range of particle to siRNA ratios was used (2 × 10^5, 4 × 10^5, 6 × 10^5, 8 × 10^5, 10 × 10^5 and 12 × 10^5/0.2 µg siRNA). The results indicate that siRNA can bind tightly to the particles when the particle amount is above 8 × 10^5. A ratio of 10 × 10^5 PCPS/0.2 µg siRNA was selected for further experiments. Furthermore, siRNA was successfully released from the carrier when treated with SDS, as illustrated in **Figure 3B**.

Next, the cellar internalization of PCPS/fluorescent control siRNA particles was evaluated in MDA-MB-231 cells. Confocal images taken after 24 hr of treatment show that the particles are effectively internalized into cells (**Figure 4**). Similarly, **Figure 5** demonstrates that 89% of cells have internalized PCPS/siRNA particles after 24 hr of incubation. Moreover, the internalization process was recorded for 12 hr (**Video 1**). These results indicate that the PCPS particles can efficiently deliver siRNA into cells. The long-term accumulation of siRNA inside the cells was also evaluated by confocal microscopy. At day 7 and day 10 the siRNA was still detectable inside the cells (**Figure 6**).

One of the most important factors to consider when developing a siRNA delivery system is the safety of the carrier[13]. PEI is known to form polyplexes with siRNA, aid in cellular uptake and trigger release from the endosome/lysosome. However, PEI can have toxic effects, due to the presence of positively charged primary amino groups in the backbone[14,15]. For instance, PEI binding to the glycocalyx on the cell surface can result in the formation of large clusters[16]. In order to eliminate this charge-induced toxicity, PEI was covalently conjugated to arginine through a bridge linker, to reduce the number of primary amino groups. The cell viability remained over 95% after 48 hr and 72 hr when particles and siRNA were used at a concentration of up to 6 × 10^5/well and 100 nM, respectively (**Figure 7A**). Next, the transfection efficacy of siRNA against the oncogene ATM was evaluated in MDA-MB-231 cells. Western blot results demonstrate that the protein levels of ATM are decreased after treatment with PCPS/ATM siRNA (**Figure 7B**). The results suggest the PCPS platform is a safe and efficient delivery system for siRNA.

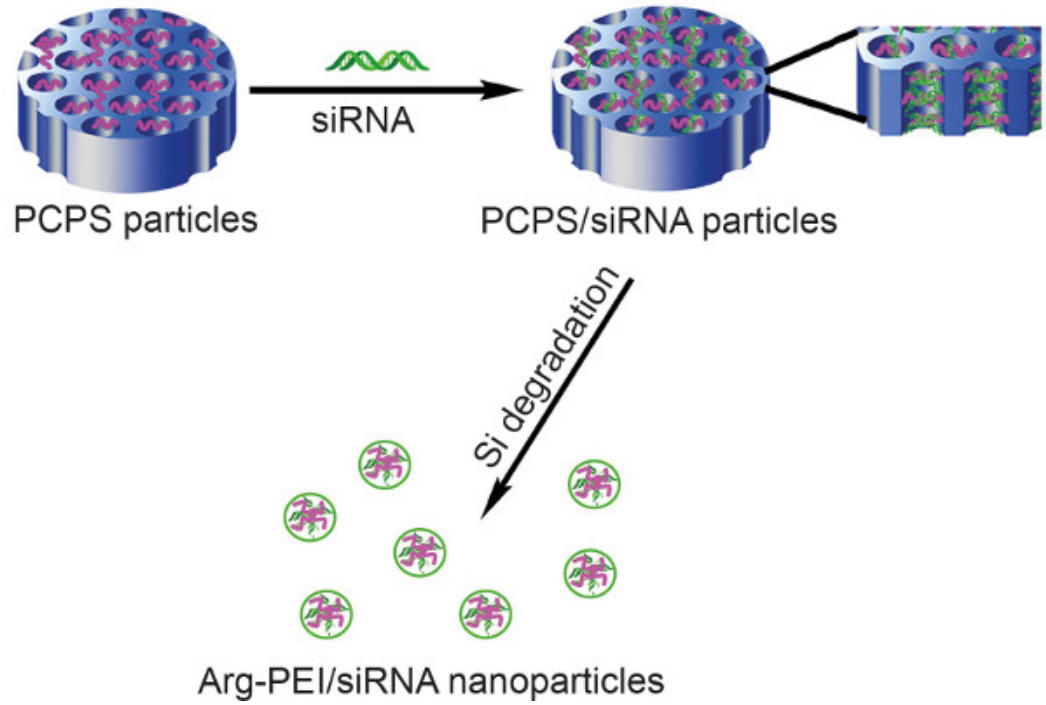

Figure 1. Schematic representation of polycation-functionalized porous silicon (PCPS) particles. Arginine (Arg)-polyethylenimine (PEI)/ small interfering RNA (siRNA) nanoparticles are formed following the degradation of silicon (Si).

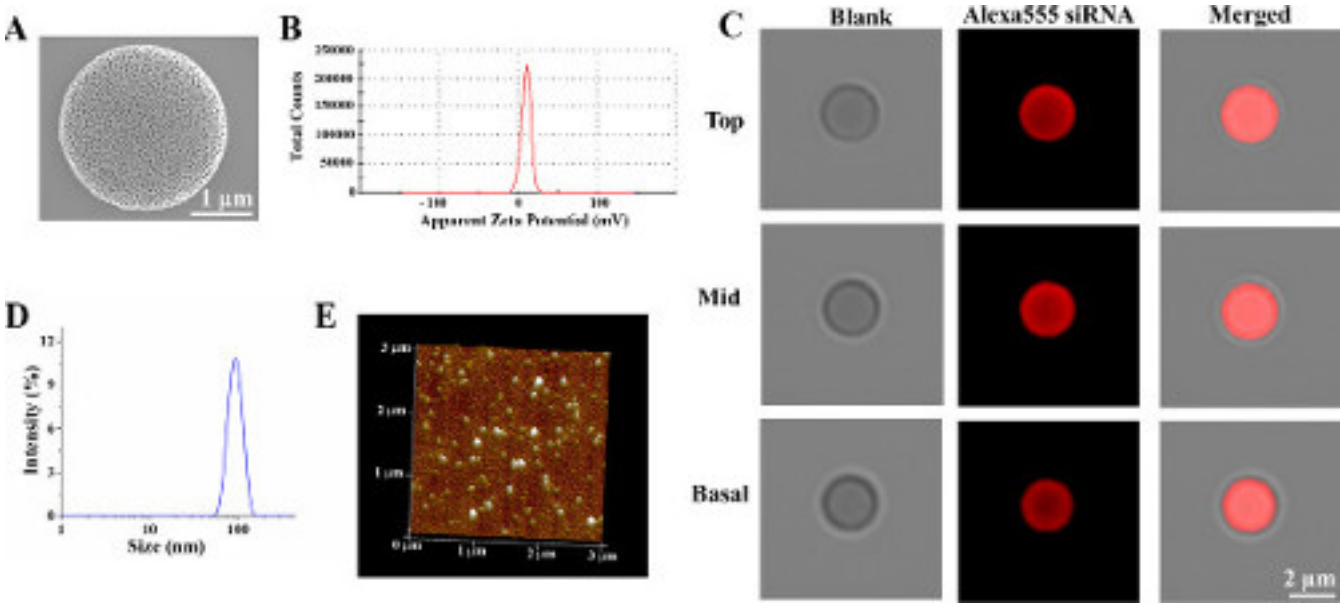

Figure 2. Characterization of PCPS particles. (**A**) Scanning electron microscopy (SEM) image of PCPS particles. (**B**) Zeta potential of PCPS particles. (**C**) Confocal images of different layers of the PCPS/fluorescent control siRNA particles. (**D**) Size distribution of the arginine Arg-PEI/ control siRNA nanoparticles released from the porous silicon microparticles. (**E**) Atomic force microscopy (AFM) images of Arg-PEI/control siRNA nanoparticles. Please click here to view a larger version of this figure.

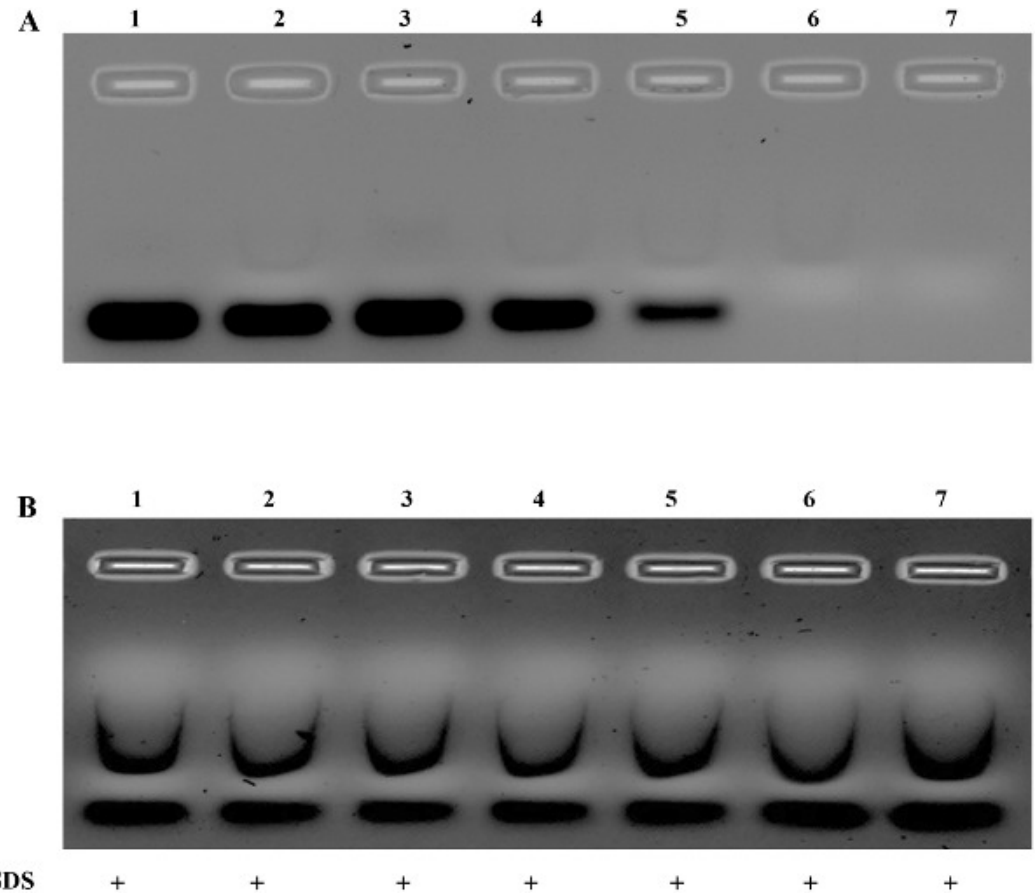

Figure 3. Agarose gel for optimization of the PCPS/siRNA delivery system. (**A**) Binding affinity between PCPS particles and control siRNA. The bands represent unbound siRNA. (**B**) siRNA release following incubation with 2% SDS for 1 hr. The bands represent total siRNA (unbound and bound). Sample 1: siRNA; sample 2: 2 × 10^5 PCPS particles/0.2 μg siRNA; sample 3: 4 × 10^5 PCPS particles/0.2 μg siRNA; sample 4: 6 × 10^5 PCPS particles/0.2 μg siRNA; sample 5: 8 × 10^5 PCPS particles/0.2 μg siRNA; sample 6: 10 × 10^5 PCPS particles/0.2 μg siRNA; sample 7: 12 × 10^5 PCPS particles/0.2 μg siRNA.

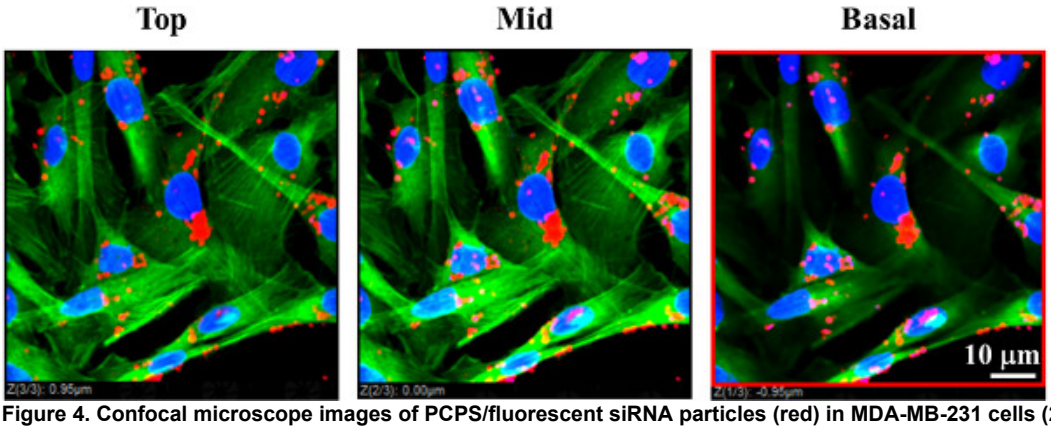

Figure 4. Confocal microscope images of PCPS/fluorescent siRNA particles (red) in MDA-MB-231 cells (24 hr incubation). The nucleus and filamentous actin was visualized with 4',6-diamidino-2-phenylindole (DAPI, blue) and phalloidin (green), respectively. Three different layers were imaged (top, mid and basal). Please click here to view a larger version of this figure.

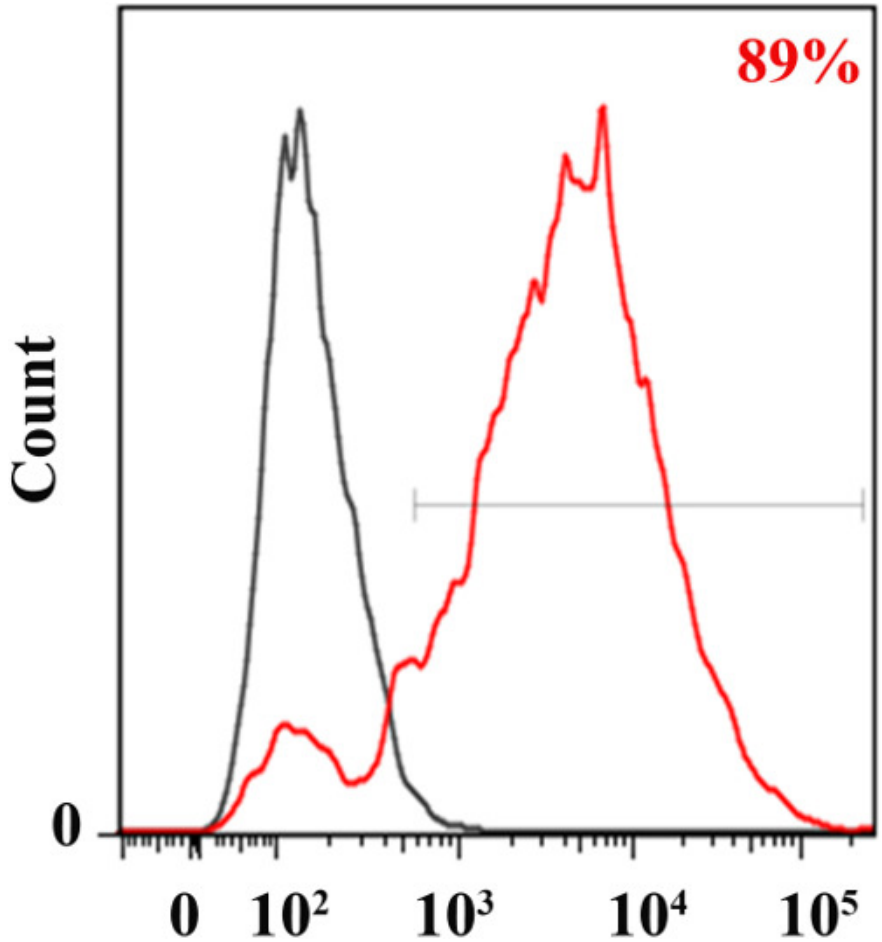

Figure 5. Quantitative flow cytometry analysis of fluorescent MDA-MB-231 cells after incubation with PCPS/control fluorescent siRNA particles. Untreated cells were used as a negative control. 89% of cells had internalized the particles.

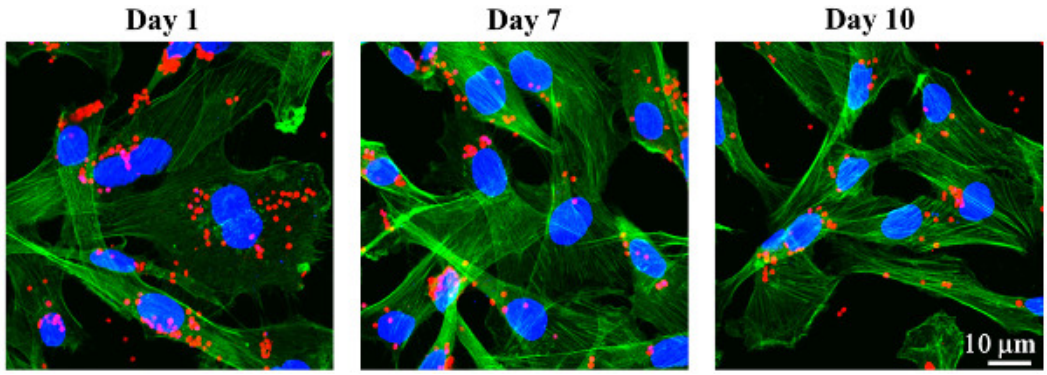

Figure 6. Confocal images of fluorescent control siRNA (red) inside MDA-MB-231 cells. Cells were incubated with PCPS/fluorescent control siRNA particles for 1 day, 7 days and 10 days. Cells were then visualized with confocal microscopy. The nucleus and filamentous actin was visualized with 4',6-diamidino-2-phenylindole (DAPI, blue) and phalloidin (green), respectively. Please click here to view a larger version of this figure.

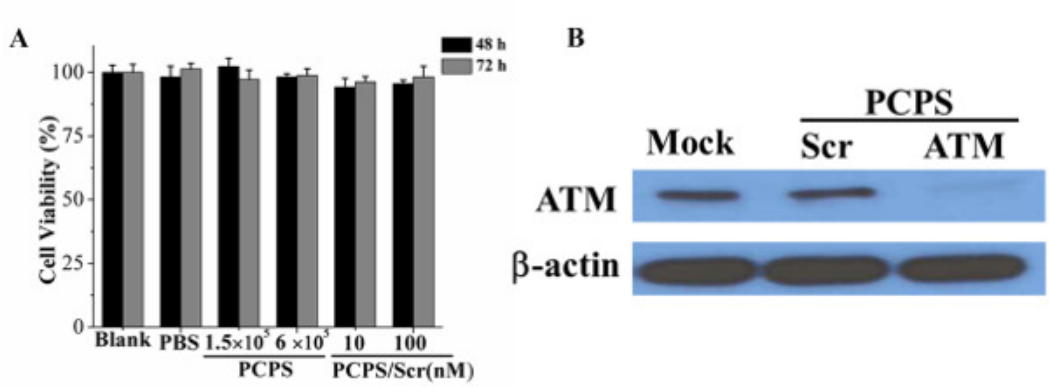

Figure 7. Cell viability and gene silencing *in vitro*. (**A**) A cell viability assay of cells incubated with PCPS particles and PCPS/control siRNA particles (Scr). Experiment was performed in triplicate and results are presented as mean ± standard deviation. Untreated cells (blank) and cells incubated with PBS were used as controls. (**B**) Western blot of PCPS/ataxia telangiectasia mutated (ATM) siRNA particles. Cells were exposed to PCPS/control siRNA (Scr) particles and PCPS/ATM siRNA particles. Untreated cells (mock) were used as a control. β-actin was used as a loading control.

Video 1. Time-dependent uptake of PCPS/fluorescent control siRNA particles in live MDA-MB-231 cells. The video was recorded for 12 hr after exposing the cells to particles.

Discussion

This protocol describes a method for the successful delivery and transfection of siRNA into cells. In particular, the delivery of siRNA is achieved by using a multifunctional platform consisting of polycation-functionalized pSi particles. The use of siRNA therapy has great potential, *e.g.*, cancer treatment, as various oncogenes can be targeted with high specificity. Therefore, there exists a demand to develop siRNA delivery vehicles, which can mitigate the challenges of siRNA therapy. In conclusion, we have outlined a protocol that shows promise for the safe and efficient delivery of siRNA. However, there are some key factors that should be taken into account when performing the described technique. For instance, an important consideration when preparing the PCPS particles is to handle the siRNA carefully, in order to avoid degradation by nucleases. In particular, clean gloves and RNAse free tubes and water should be used at all times when working with siRNA. If the siRNA transfection efficacy is low, a spray that removes RNAse contamination can be used to spray gloves and working areas. In addition, if the transfection is unsuccessful, the siRNA should be tested with a commercial transfection reagent, to determine whether the problem is caused by the siRNA or the particles. Another critical consideration for successful implementation of the PCPS/siRNA delivery system is to take care when performing centrifugation and washing steps. Namely, the supernatant should be fully discarded to remove excess reagents that are required throughout the particle preparation steps.

An important advantage of the PCPS/siRNA delivery system is safety. Several siRNA transfection agents have a high cationic charge, which contributes to cellular toxicity[13,15]. Indeed, most commercial protocols indicate that the reagents should be incubated with the cells for only a few hours, to avoid cell death. On the contrary, cells can be exposed to the PCPS particles for several days without any signs of toxicity, as is evident from the cell viability results. The PCPS particles can bind siRNA with high affinity due to the presence of PEI. However the charge-induced toxicity of PEI is prevented by the unique setup of the delivery platform. Especially the covalent binding of Arg to PEI and the encapsulation of PEI inside the Si pores contribute to reduced toxicity. Another advantage of the PCPS platform is that siRNA transfection can take place in the presence of serum. Other existing methods usually require the use of serum-free cell culture media, thereby adding additional steps to the transfection process and potentially interfering with regular cell signaling pathways.

An additional benefit of the PCPS system is that it does not require any modification of the siRNA molecules. While some current methods require complicated conjugation steps to stabilize the siRNA or to enable cellular internalization[13], the PCPS system relies on simple mixing for siRNA loading. The binding to PEI and the pores in the Si material provide a protective environment for the siRNA, thereby reducing contact with nucleases. The PCPS particles can be stored for prolonged periods of time as dried material or in isopropyl alcohol. Moreover, if the PCPS/siRNA particles are lyophilized they can be stored for at least three months at 4 °C. The lyophilized PCPS particles should be suspended in RNase free water just before transfection, and should not be stored in solution. Finally, the PCPS platform permits sustained release of siRNA, as previously reported[11], consequently increasing the time period in which target genes remain suppressed. Accordingly, after cellular internalization, the Si particles gradually degrade, resulting in the formation of Arg-PEI/siRNA nanoparticles. Subsequently, the siRNA is slowly released in the cytoplasm, where it can bind to messenger RNA (mRNA), thereby exerting biological activity. Although, PCPS particles provide several advantages in comparison to existing methods for siRNA delivery, a limitation of the technique is that non-functionalized pSi microparticles are required as a starting material. While the particles can be synthesized using photolithography and electrochemical etching[17], these techniques are not readily available at all institutions.

Disclosures

The authors declare that they have no competing financial interests.

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