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# Polycation-functionalized nanoporous silicon particles for gene silencing on breast cancer cells

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ABSTRACT

Nanoporous silicon particles (pSi), with a pore size in the range of 20–60 nm, were modified with polyethyleneimine (PEI) to yield pSi–PEI particles, which were subsequently complexed with siRNA. Thus, pSi–PEI/siRNA particles were fabricated, with the PEI/siRNA nanocomplexes mainly anchored inside the nanopore of the pSi particles. These hybrid particles were used as carriers to deliver siRNA to human breast cancer cells. Due to the gradual degradation of the pSi matrix under physiological conditions, the PEI/siRNA nanocomplexes were released from the pore interior in a sustained manner. Physicochemical characterization revealed that the released PEI/siRNA nanocomplexes exhibited well-defined spherical shape and narrow particle size distribution between 15 and 30 nm. Gene knockdown against the ataxia telangiectasia mutated (ATM) cancer gene showed dramatic gene silencing efficacy. Moreover, comprehensive biocompatibility studies were performed for the pSi–PEI/siRNA particles both *in vitro* and *in vivo* and demonstrated that the pSi–PEI particles exhibited significantly enhanced biocompatibility. As a consequence, PEI-modified porous silicon particles may have substantial potential as safe and effective siRNA delivery systems.



## 1. Introduction

In order to reap the full benefits of RNA interference (RNAi)-based therapy, effective siRNA delivery systems are highly desirable. It has been evidenced that non-viral siRNA delivery systems are superior to their viral counterparts, due to easy preparation, lower cost, enhanced biocompatibility, and improved biosafety [1–9]. In particular, mesoporous silica nanoparticles (MSNs), with a typical pore size in the range of 2–10 nm, have demonstrated promise as carriers for nanomedicine, including nucleic acid, among others [10–12]. Nano-constructs, typically formed via self-assembly between oppositely charged species, i.e., a nucleic acid and a cationic polymer, are installed or adsorbed outside the mesopore as "nanogate" to prevent small molecules (i.e., anti-cancer agent) entrapped inside the mesopore from leaking out. Upon application of external stimuli, the nanogate dissociates releasing the small-molecule cargo inside the mesopore. It is recognized that the small pore of MSNs may hinder the efficient loading of larger biomolecules (i.e., nucleic acid or protein). Therefore, most of the studies, in which MSNs were used for nucleic acid delivery, have been focused on surface coating of the MSNs with a cationic polymer (i.e., PEI), allowing for complexation with anionic nucleic acid [11,12]. It should be noted that since the polymer/nucleic acid complexes were adsorbed or anchored on the outer surface of the MSNs, these nanoconstructs, which were self-assembled via electrostatic interaction, might be vulnerable to enzymatic degradation or even compromised upon injection in the systemic circulation.

In an effort to address such challenges induced by the small-pore size, Na et al. recently managed to expand the pore size of the as-prepared small-pore MSNs, i.e., increased from 5 to 23 nm, by means of post-synthesis treatment of the small-pore MSNs [13]. Comparisons were made in terms of their applications as siRNA delivery systems between the two types of MSNs with different pore sizes. Results showed that increasing the pore size of MSNs could be a useful strategy towards the improvement of MSN-based siRNA delivery for *in vivo* applications.

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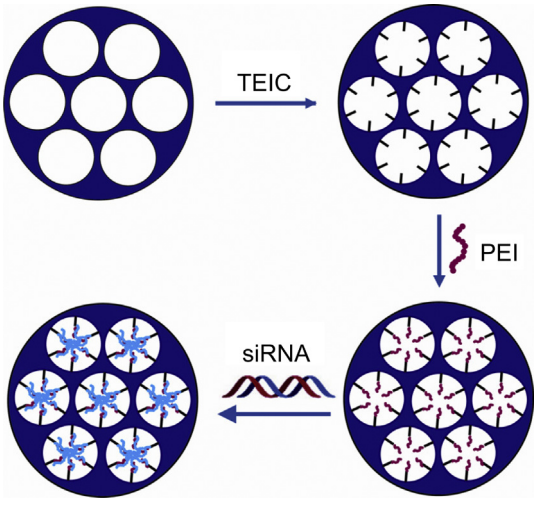
Over the past few years, we have developed a series of nanoporous silicon particles (pSi) with a much larger pore size (i.e., with an average diameter of 20–60 nm) and have utilized such particles as multi-stage vectors (MSVs) for systemic delivery of therapeutic or diagnostic agents, including siRNA [14–21]. Due to the bigger pore size, nanoconstructs packaged with therapeutics could be readily loaded inside the pore interior of the pSi particles to achieve sustained delivery to tumor tissues. In a typical MSV approach, charged nanoliposomes packaged with small molecule drugs or therapeutic siRNA are loaded into the pore interior of the pSi particles via electrostatic interaction and capillary force. Once inside the body, the pSi particles (or stage 1 particles) are gradually degraded and nanoliposomes (or stage 2 particles) are released from the pSi particles, thus achieving multi-stage release. This delivery system has such advantages as enhanced loading efficiency and easy tunability in particle shape and size, allowing for efficient encapsulation of nano-sized species into the MSV in order to shield them from contacting with the unintended organs or cells, which leads to minimal toxicity and enhanced efficacy.

Moreover, investigating the effects of shape and size on the biological properties both *in vitro* and *in vivo* showed that in comparison to hemi-spherical pSi particles [22], discoidal pSi particles exhibited enhanced properties towards their applications as effective carriers in cancer therapy, as evidenced from their increased surface area, improved biodistribution in multiple animal tumor models, among others. In view of the complex biological environment, i.e., presence of numerous charged species in the plasma and in tumor interstitium, it would be very useful to develop a loading strategy in which the siRNA-containing nanocomplexes are anchored inside the nanopores of pSi particles in order to minimize the interaction between the siRNA-containing nanocomplexes and the charged biological species upon systemic administration of the resultant pSi particles. Upon gradual degradation of the pSi matrix, the siRNA-containing nanocomplexes can be released in a sustained manner such that favorable pharmacokinetics could be achieved. Additional feature rendered by such a delivery system is its versatility for multiple therapies, which is of dramatic clinical significance [23].

Herein, we describe a platform, in which a cationic polymer, namely polyethyleneimine (PEI), is readily conjugated to the pore interior of pSi particles via straightforward chemistry, followed by electrostatic complexation with anionic siRNA to form PEI/siRNA nanoparticles (Scheme 1). PEI has been widely used as non-viral delivery systems for nucleic acids [24,25]. Upon gradual degradation of the pSi matrix under physiological conditions, PEI/siRNA nanoparticles are released from the nanopore confinement. The resulting nanoparticles are subsequently internalized into cells leading to gene silencing. The ataxia telangiectasia mutated (ATM) gene was chosen as the target gene to test this delivery system. We and others have previously shown that ATM plays an important role in cancer therapy [17,26].




**Scheme 1.** Synthesis of pSi–PEI/siRNA hybrid particles.

## 2. Materials and methods

### 2.1. Materials

All reagents and medium were obtained from Sigma Aldrich (USA), Lonza, or Promega (USA), and used without further purification. RNase-free $H_2O$ was supplied by Fisher Scientific (USA). siRNAs were synthesized by Thermo Scientific. All other chemicals and reagents were of analytical grade and were used as received.

### 2.2. Preparation of pSi particles

pSi particles were fabricated by electrochemical etching of silicon wafers in the Microelectronics Research Center at The University of Texas at Austin as previously described [27]. The pSi particles were oxidized with $H_2O_2$ (30%) at 100 °C for 2 h to the –OH functionality on the surface. Subsequently, the oxidized pSi particles were reacted with 3-(triethoxysilyl)propyl isocyanate (TEIC) to yield the –NCO functionality [28], which was subjected to conjugation of PEI in anhydrous ethanol. The mixture was rinsed with ethanol and centrifuged twice to remove unreacted chemical or PEI from the pSi particles.

### 2.3. Particle characterization

Zeta potential measurements and dynamic light scattering (DLS) were carried out using a Zeta Sizer Nano ZS (Malvern Instrument, UK). Particles were dispersed in PB buffer (pH 7.4) at a concentration of 0.5 mg/mL. The samples were mixed well by sonication for 10 s before analysis. Morphological observation was performed using Bruker MultiMode atomic force microscopy (Bruker, USA). Particle density was measured using a Multisizer 4 Coulter Particle Counter (Beckman Coulter, USA). Prior to the analysis, the samples were dispersed in the balanced electrolyte solution (ISOTON® II Diluent, Beckman Coulter, USA) and sonicated for 10 s to ensure a homogenous dispersion. Absorbance and fluorescence measurements were performed with BioTek Synergy H4 hybrid multi-mode microplate reader (BioTek, USA) using a Take3 Micro-Volume plate (BioTek, USA). Morphological studies were performed using scanning electron microscopy (FEI Nova NanoSEM 230) operated at 20 keV or a transmission electron microscopy (JEOL 2010) equipped with a CCD camera and operated at 120 keV.

### 2.4. In vitro transfection

MDA-MB-231 cells were seeded in 6-well microplates at a density of $2 \times 10^5$ cells/well and allowed to attach overnight in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). After the attachment, the culture medium was replaced with fresh DMEM without FBS. The as-prepared pSi–PEI/siRNA particles were re-dispersed with DMEM to desired concentrations. 100 μL of the diluted sample solution was added to each well, and the cells were incubated at 37 °C for 60 h under 5% $CO_2$ atmosphere. As a control, siRNA transfected with a commercial transfection reagent INTERFERin (Polyplus Transfection, France) was used, by following the manufacturer's instructions.

### 2.5. Confocal laser scanning microscopy (CLSM) observation

Confocal images were acquired at the ACTM Core facility of TMHRI, using a Fluoview 1000 laser scanning fluorescence microscope (Olympus, Japan) equipped with an oil-immersion 100× numerical aperture PlanS Apo objective. The cells were seeded on 35-mm dishes with a cover glass bottom (MatTek Corporation, Ashland, MA). In order to visualize the hybrid particles, Alexa Fluor 555-labeled siRNA was used. Identical protocol was employed for the siRNA transfection as that for the above-mentioned transfection experiments. After transfection for desired time intervals, the cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS. The nuclei and the endosomes/lysosomes were stained with DAPI and LysoTracker Green, respectively. Excitation wavelengths were 405, 488, and 543 nm, for DAPI, LysoTracker Green, and Alexa Fluor, respectively.

### 2.6. Western blot

MDA-MB-231 cells were seeded in 6-well plates ($2 \times 10^5$ cells per well). Identical cell culture conditions and siRNA transfection protocols as described above were followed. 60 h after the transfection, the cells was rinsed with PBS and