Supplementary Information

Porous silicon-graphene oxide core-shell nanoparticles for targeted delivery of siRNA to the injured brain

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Experimental details

Materials. Single-crystalline highly doped p-type silicon wafers (boron-doped, ~ 1 mΩ cm resistivity), polished on the (100) face, were purchased from Virginia Semiconductor, Inc. 3-(ethoxydimethyl)-propylamine silane, graphene oxide (dispersion in water, 2 mg/mL), and paraformaldehyde were purchased from Sigma-Aldrich Chemicals, inc. Succinimidyl carboxymethyl ester-polyethylene glycol-maleimide (SCM-PEG-Mal, MW 5000) and succinimidyl carboxy methyl ester-polyethylene glycol (SCM-PEG) were purchased from Laysan Bio, inc. All chemicals were used as received without further purification. RNase free water, phosphate buffered saline (PBS, pH 7.4), fetal bovine serum, Opti-MEM, Lipofectamine RNAiMAX transfection agent, Quant-iT™ RNA assay kit, and RNase A were purchased from Life Technologies. Eagle's Minimum Essential Medium (EMEM) was purchased from ATCC. Small interfering RNA against peptidylprolyl isomerase B (siPPIB) and luciferase (siLuc) were purchased from Dharmacon, inc. A 1:1 mixture of siPPIB(1) and siPPIB(2) were used for gene silencing. The sequences for the sense strands were:

siPPIB(1): 5'-CAAGUUCCAUCGUGUCAUCdTdT-3'

siPPIB(2): 5'-GAAAGAGCAUCUAUGGUGAdTdT-3'

siLuc: 5'-CUUACGCUGAGUACUUCGAdTdT-3'

The RVG peptide with N-terminal cysteine residues, (CCGG)YTIWMPENPRPGTPCDIFTNSRGKRASNG, was obtained from CPC Scientific, Inc. FAM-RVG, which has 5-carboxyfluorescein (5-FAM) attached *via* an amide bond to the N-terminal cysteine residue on RVG, was also purchased from CPC Scientific, Inc. The FAM-RVG construct used for confirmation of peptide conjugation to the nanocarriers.

Preparation of porous silicon nanoparticles. Porous silicon nanoparticles (pSiNPs) were prepared by electrochemical perforation etching of silicon wafer, as described previously.[1] The silicon wafer was anodically etched in an electrolyte consisting of 3:1 (by volume) of 48% aqueous HF:ethanol. Etching was carried out in a Teflon etch cell that exposed 8.0 cm^2 of the polished surface of the Si wafer to the electrolyte, using a coil of platinum wire as the counter electrode. The silicon wafer was contacted on the backside with a strip of aluminum foil. The etching waveform

consisted of a square wave in which a lower value of current density of 50 mA/cm^2 was applied for 1.8 sec, followed by a higher value of current density of 400 mA/cm^2 for 0.36 sec. This waveform was repeated for 140 cycles, generating a perforated porous silicon film with alternating layers of high and low porosity. The resulting porous nanostructure was removed from the silicon substrate by applying a current density of 3.7 mA/cm^2 for 250 sec in an electrolyte consisting of 1:30 (by volume) of 48% aqueous HF:ethanol. The resulting pSi layers were rinsed several times with absolute ethanol, then placed in absolute ethanol (7 mL) in a vial and fractured into nanoparticles by ultrasonication (50T ultrasonic bath, VWR International) for 15 hr. The nanoparticles were isolated by centrifugation (Eppendorf Centrifuge Model 5804) at 15,000 rpm for 12 min and then redispersed in aqueous borax solution (1 mM) for 2.5 hr to activate photoluminescence.[2] The nanoparticles were then isolated by centrifugation (Eppendorf Centrifuge Model 5804) at 15,000 rpm for 12 min, the supernatant removed and the pellet rinsed 3 times with absolute ethanol and deionized water, respectively. The procedure typically yielded 9 mg of nanoparticles.

Preparation of positively charged graphene oxide (GO) nanosheets. The as-purchased aqueous GO suspension was subjected to ultrasonic fracture (50T ultrasonic bath, VWR International) overnight and subsequently centrifuged (15,000 rpm, 12 min) to obtain GO nanosheets. Afterwards the desired GO nanosheets (~ 200 nm) were still stably dispersed in the supernatant, while larger sized sheets settled. The GO nanosheets were separated and further modified *via* EDC-mediated reaction with ethylenediamine to impart a net positive charge to the sheets:[3] 1 mL of ethylenediamine and 100 mg of EDC were added to the GO nanosheet dispersion (3 mL), and the mixture was stirred overnight. The resulting GO nanosheets were subjected to dialysis for 2 days using a 12-14 kD MWCO membrane (Spectrum Laboratories, Inc.) to completely remove residual reagents. The resulting GO nanosheets were used in the pSiNP wrapping procedure.

Preparation of siRNA-loaded nanocarrier formulations. An aliquot (1 mL) of pSiNPs (2 mg/mL in ethanol) was mixed with 20 μL of 3-(ethoxydimethyl)-propylamine silane by vortexing overnight at room temperature. The amine-terminated nanoparticles were rinsed three times with ethanol and RNase free water.

siRNA was loaded into the cationic pSiNPs by mixing with siRNA stock solution. The concentration of free siRNA was determined by measuring UV absorbance at 260 nm using a spectrometer (NanoDrop 2000, Thermo Fisher Scientific) and comparing it to a standard curve of siRNA. The suspension was centrifuged to separate the free siRNA from the siRNA-loaded pSiNP pellet. To prepare GO-encapsulated pSiNPs (GO-pSiNPs), an aqueous suspension of GO nanosheets was added to an aqueous suspension of the siRNA-loaded pSiNPs, and the mixture was stored at 4 °C for 1 hr to complete the electrostatic wrapping process. The GO-pSiNPs were then collected by centrifugation to remove any unassociated GO nanosheets remaining in suspension. A solution (1 mL) of either the SCM-PEG-Mal or SCM-PEG linker (5 mg/mL, in ethanol) was added to the GO-pSiNPs and stored at 4 °C for 2 hr. The succinimidyl carboxymethyl ester group reacts with the primary amine group on the GO-pSiNPs. Unbound PEG linker molecules were removed from the PEGylated nanocarriers by centrifugation (15000 rpm, 12 min). The PEG-terminated GO-pSiNPs were used as the non-targeted control particles, and the maleimide-activated nanocarriers were further mixed with 500 μL of the RVG peptide solution (1 mg/mL in RNase free water) and incubated at 4 °C for 2 hr to conjugate the peptide via a free cysteine residue at the terminal group of the peptide.[4] Peptide conjugation was confirmed from the fluorescence spectrum of the FAM labeled-peptide.

Characterization of pSiNPs. Transmission electron microscope (TEM) images were obtained on JEOL-1200 EX II operating at 120 kV. A Malvern Instruments Zetasizer ZS 90 was used to determine the hydrodynamic size (by dynamic light scattering, DLS) and zeta potential of the nanoparticles. Nitrogen adsorption-desorption isotherms were obtained on dried pSiNPs at a temperature of 77K with a Micromeritics ASAP 2020 instrument. Photoluminescence and fluorescence spectra were obtained using an Ocean Optics QE65 pro spectrophotometer. Raman spectra were obtained using a Renishaw inVia Raman microscope with 532 nm laser excitation. Concentration of siRNA was determined by measuring absorbance at 260 nm using a spectrometer (NanoDrop 2000, Thermo Fisher Scientific) based on the OD_{260} standard curve of siRNA.

In vitro gene silencing. Neuro-2a neuroblastroma cells (ATCC, CCL-131) were cultured in Eagle's Minimum Essential Medium (EMEM) containing 10% fetal

bovine serum (FBS). Cells were seeded in 24-well plate at 6×10^4 cells per well the day before the assay. The culture media were replaced with serum-free transfection media (Opti-MEM), and the nanocarrier formulations (resuspended in Opti-MEM, equivalent to 200 nM siRNA) were incubated with the cells for 4 hr. As a positive control for gene silencing, siRNA/Lipofectamine RNAiMAX complexes were prepared according to the manufacturer's protocol. After 4 hr treatment, transfection media were replaced with the complete EMEM, and the cells were further incubated at 37 °C. After 48 hr of incubation, the cells were harvested and lysed, and total RNA was extracted using an RNeasy mini kit (QIAGEN), according to the manufacturer's protocol. Reverse transcription reaction was performed using iScript cDNA Synthesis Kit (Bio-Rad). 1 μg of extracted RNA was reverse transcribed into cDNA according to the manufacturer's protocol (iScript cDNA Synthesis Kit, Bio-Rad). Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis was performed to examine PPIB mRNA expression with an Mx3005P qPCR system (Agilent). The synthesized cDNA was subjected to RT-qPCR using the SYBR Green PCR Mater Mix (Bio-Rad). Samples were normalized to levels of hypoxanthine phosphoribosyltransferase (HPRT) for qPCR analysis. Primer sequences used for qPCR were:

PPIB forward: GGAAAGACTGTTCCAAAAACAGTG; PPIB reverse: GTCTTGGTGCTCTCCACCTTCCG.

HPRT forward: GTCAACGGGGGACATAAAAG; HPRT reverse: CAACAATCAAGACATTCTTTCCA.

Cellular uptake. Cells were seeded onto coverslips (Corning Biocoat Poly-D-Lysine, 12 mm) and cultured overnight in the complete EMEM. The cell culture media were replaced with Opti-MEM, and the nanocarrier formulations were added as described above. After 2 hr of incubation with nanocarriers, the cells were washed three times with PBS, fixed with 4% paraformaldehyde, and mounted for fluorescence microscope imaging (Zeiss LSM 710 NLO Confocal Microscope). To quantitatively demonstrate cellular uptake of Dy547-labeled siRNA, cells were harvested by trypsinization and subjected to flow cytometric analysis with a LSR Fortessa (BD Biosciences). Forward and side-scatter gates were set to exclude debris. Data were analyzed using BD FACSDiva software.

RNase treatment and siRNA stability assay. The siRNA-loaded nanocarriers (2 mg/mL, 12% by mass loaded oligonucleotide) were incubated at 37 °C in PBS solutions containing various concentrations of RNase A (total volume 1 mL) for 1 hr and then separated from the RNase-containing solutions by centrifugation. The siRNA was then released from the pSiNPs by treatment with aqueous potassium hydroxide (50 μM, 150 μL) to dissolve the silicon skeleton (pH ~ 9.5). The solution was then analyzed using a Quant-iT RNA assay kit (Life Technologies, inc.) and UV absorbance. *In vitro* gene silencing experiments were carried out by incubating Neuro-2a cells with the nanocarriers in RNase-containing serum media (EMEM with 10% FBS).

In vivo siRNA delivery. All studies in mice were approved by the MIT Institutional Animal Care and Use Committees (IACUC). Brain injuries were created by removing a 5 mm diameter portion of the skull and inducing 3 mm deep wounds 1 mm apart in a 3x3 grid using a 19G needle on the right hemisphere of the brain. After induction of the injuries, the missing part of the skull was replaced. The nanocarriers (200 μg) were resuspended in PBS (100 μL) and administered via tail-vein injections 6 hr post injury. The mice were sacrificed 2 hr following injection and major organs were harvested for downstream analysis. The organs were imaged using an IVIS instrument (Xenogen), Odyssey CLx (Li-Cor Biosciences) and custom-built GLISiN (Gated Luminescence Imaging of Silicon Nanoparticles) system.[5]

Statistical Analysis. All data represent mean values ± standard deviation. Statistical significance was evaluated using two-tailed heteroscedastic Student's *t* test. Unless otherwise stated, the threshold of statistical significance was set to $p < 0.05$.

Figure S1. (a) Nitrogen adsorption-desorption isotherm and (b) BJH pore size distribution of a typical pSiNP preparation used in this work. Measurements made on oxidized porous Si nanoparticles. Inset: Plan-view scanning electron microscope (SEM) image of the pSi film before ultrasonication.

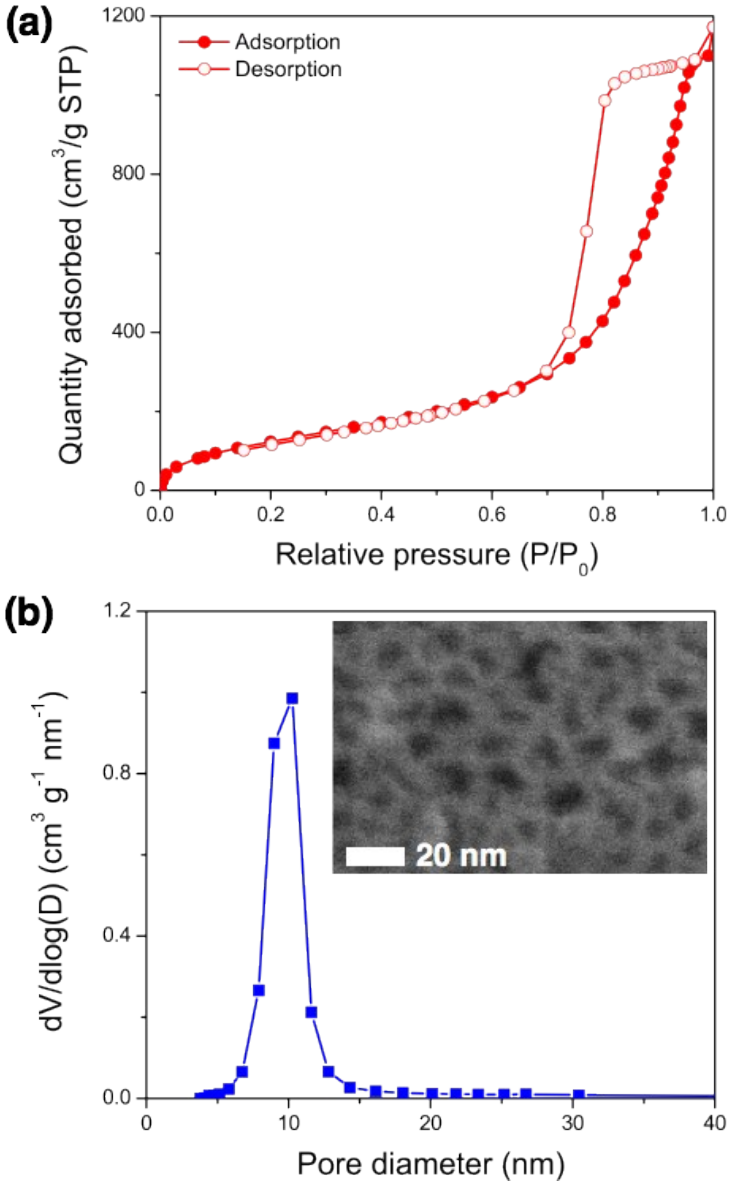
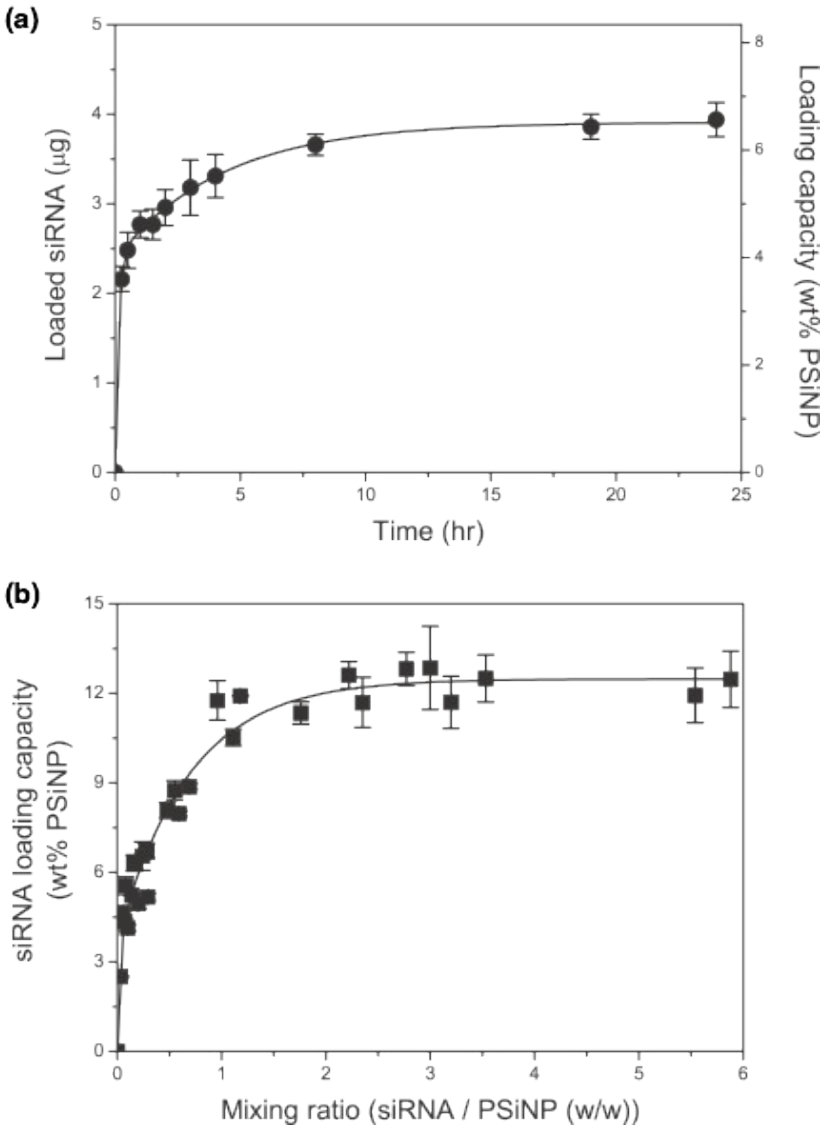


Figure S2. Loading and release characteristics of siRNA-loaded pSiNPs and GO-pSiNPs. Mass loading of siRNA into pSiNPs as a function of incubation time (a) and mixing ratio (b). Plot in (a) represents samples loaded at a siRNA/pSiNP mixing ratio of 0.12 (w/w). Plot in (b) represents samples loaded for fixed incubation time of 24 hr.



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