Supporting Information for

Single siRNA Nanocapsules for enhanced RNAi delivery

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Materials and Methods

Materials All chemicals were purchased from Sigma-Aldrich unless otherwise noted, and were used as received.

siRNA sequence. RNA oligonucleotides were ordered from Sigmaaldrich. The siRNA duplex against luciferase was ordered by using both the antisense (5'-UAUCGAAGGACUCUGGCACdTdT-3') and sense (5'-GUGCCAGAGUCCUUCGAUAdTdT-3) strands. The siRNA duplex against CCR5 was ordered by using both the antisense (5'-GGUGUAAACUGAGCUUGCUCUU-3') and sense (5'-GAGCAAGCUCAGUUUACACCUU-3') strands. The nontargeting control siRNA duplx was ordered by using both the antisense (5'-UAGCGACUAAACACAUCAAUU-3') and sense (5'UUGAUGUGUUUAGUCGCUAUU-3').

Instrument TEM images of nanocapsules were obtained on a Philips EM120 TEM at 100000X. Before observation, siRNA nanocapsules were negatively stained using 1% pH 7.0 phosphotungstic acid (PTA) solution. Zeta potential and particle size distribution were measured with a Malvern particle sizer Nano-ZS. Fluorescent images of cells were obtained with either Zeiss Axio Observer.Z1 fluorescence microscope or Leica TCS SP MP Inverted Confocal Microscope. Cellular fluorescent intensity distribution was determined with Becton Dickinson FACScan Analytic Flow Cytometer. A 488 nm argon laser was used as the excitation light. UV–vis absorption spectra were obtained on a Thermo Scientific NanoDrop 2000 at wavelengths between 190 and 364 nm, using a 50uL quartz cuvette.

Synthesis of Acryl-spermine The preparation of acryl-spermine was achieved by reacting spermine with acrylic acid, hydroxysuccinimide ester (NAS). Briefly, spermine (100 mg) and NAS (80 mg) were dissolved in 1 mL chloroform, respectively. NAS solution was then added into spermine solution gradually at room temperature under vigorous stirring. After overnight reacting, the mixture was filtered to remove by-products. The filtrate was then dried by rotary evaporation, followed by re-dispersing with ddH2O. After removal of insolublesubstance, the solution was lyophilized. Finally, acryl-spermine was purified by process TLC.

^{1H} NMR (400 MHz, D_2O): 6.26 (m, 1H, CH_2=CHCO), 5.76 (m, 2H, CH_2=CHCO), 3.23 (m, 2H, CONH-CH_2), 2.68 (m, 10H, CH_2--NH-CH_2 and CH_2-NH_2), 1.70 (m, 4H, NH-CH_2-CH_2), 1.15 (m, 4H, CH_2-$(CH_2)_2$-CH_2)

Synthesis of siRNA Nanocapsule siRNA was dissolved in 20uL Rnase-free water at 20uM. Then a specific amount of acryl-spermine, tris-acrylamide and glycerol dimethacrylate (molar ratio = 5:5:1) dissolved in 0.5mL deoxygenated

and deionized water was mixed with siRNA in the microcentrifugetube (final molar ratio of siRNA to acryl-spermine=1:220). Radical polymerization from the surface of siRNA was initiated by adding 2uL of 1% ammonium persulfate solution and 1uL of 5% N,N,N',N'-tetramethylethylenediamine solution. The reaction was allowed to proceed for 60 min in a nitrogen atmosphere.

Synthesis of BSA Nanocapsule BSA was dissolved in 20uL Rnase-free water at 20uM. Then a specific amount of acryl-spermine, tris-acrylamide and glycerol dimethacrylate (molar ratio = 5:5:1) dissolved in 0.5mL deoxygenated and deionized water was mixed with BSA in the microcentrifugetube (final molar ratio of BSA to acryl-spermine=1:220). Radical polymerization from the surface of BSA was initiated by adding 2uL of 1% ammonium persulfate solution and 1μL of 5% N,N,N',N'-tetramethylethylenediamine (final molar ratio of BSA to acryl-spermine=1:240). The reaction was allowed to proceed for 60 min in a nitrogen atmosphere.

Characterizing of Nanocapsules TEM and dynamic light scattering was used to determine the size and size distribution of single-siRNA nanocaspules. The electrophoretic light scattering was used to investigate the zeta potential of nanocapsules. The stability of siRNA and single-siRNA nanocapsule against Rnase and serum has been compared. Degradation of siRNA nanocapsules was investigated in 50 mM sodium acetate buffer (pH 5.4) and 50mM HEPES buffer (pH 7.4).

pH Titration of Nanocapsule The buffering capacity of the nanocapsule was measured by acid–base titration. The nanocapsule solutions (8.3 mM in terms of total molar concentration of ioniziable amine groups), which were initially adjusted to pH 10, were titrated with 0.01 M HCl. The pH profiles were recorded at room temperature.

***In vitro* Intracellular Delivery of the siRNA Nanocapsules in CWR cells stably expressing luciferase** We tested the siRNA delivery efficiency in CWR cells stably expressing luciferase with these nanocapsules. CWR cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine fetal serum (BFS) and 1% penicillin/streptomycin. CWR Cells (5000 cells/well, 96-well plate) were seeded the day before adding the siRNA nanocapsules. siRNA nanocapsules with different concentrations were added into the serum-free medium or 50% human serum medium. After incubation at 37 °C for 4 hours, the cells were washed three times with phosphate buffered saline (PBS). Then mediums were changed to DMEM with 10% Bovine Fetal Serum. After 48 h, the luciferase activity was determined using a 96-wells plate reader.

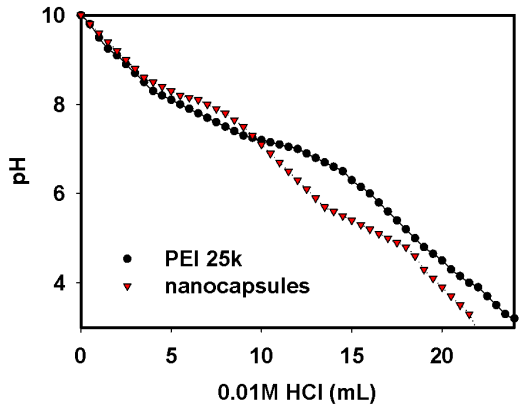
In vitro Intracellular Delivery of the siRNA Nanocapsules in HEK-293T cells In HEK-293T cells, cellular internalization studies were assessed via fluorescence microscopic technique and fluorescence-activated cell sorting (FACS). 293T cells were cultured in Dulbecco's modified Eagle's medium

(DMEM) supplemented with 10% bovine fetal serum (BFS) and 1% penicillin/streptomycin. 293T Cells (10000 cells/well, 24-well plate) were seeded the day before adding the siRNA nanocapsules. Cells were treated with CCR5-mCherry plasmid (1ug per well) and lipofectamine (1uL per well) complex for 2hrs at 37 ^{o}C in different cell culture medium. Then cells were washed with PBS and treated with siRNA nanocapsules or siRNA lipofectamine complex at 200nM in serum-free medium or 50% human serum medium. Finally, mediums were changed to DMEM with 10% Bovine Fetal Serum. After 48 h, the images were taken or flow cytometry was run. The relative CCR5-mCherry expression (%) was calculated from the mean fluorescence.

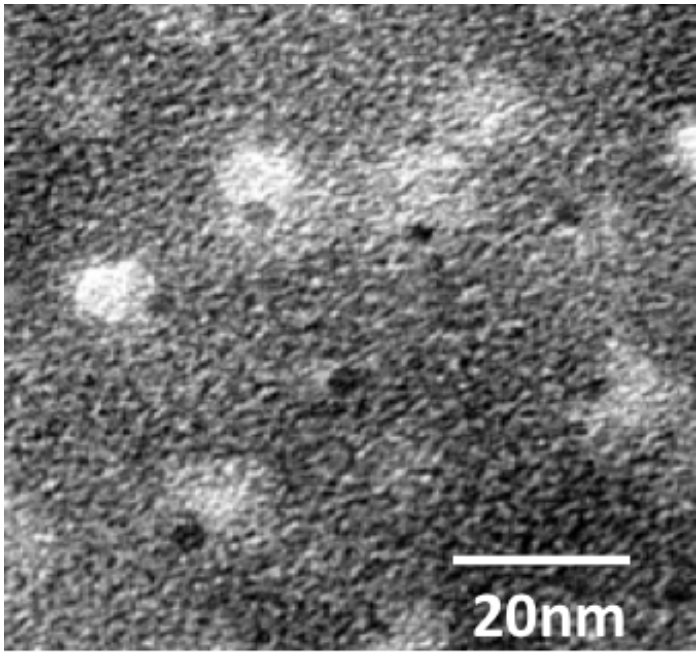
Cell viability assay The toxicity of the nanocapsules was assessed by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using native proteins as control. CWR cells (5000 cells/well) were seeded on a 96-well plate the day before exposure to the nanocapsules. Nanocapsules with different concentrations were incubated with the cells for 4 hours, removed from the mixture, and incubated with fresh media for 24 hrs. The MTT solution (20 µL) was added to each well and incubated for 3 h. The medium was then removed and 100 µL DMSO was added onto the cells. The plate was placed on a shaking table, 150 rpm for 5 min to thoroughly mix the solution, and then absorbance readings were measured at 560 nm. Untreated cells were used as the 100% cell proliferation control.

Figures for Supporting Information (SI)

SI Figure 1. Titration profiles of nanocapsules (triangle) and PEI25k (circle). Solution were titrated from pH 10 to pH 3 with 0.01 M HCl.

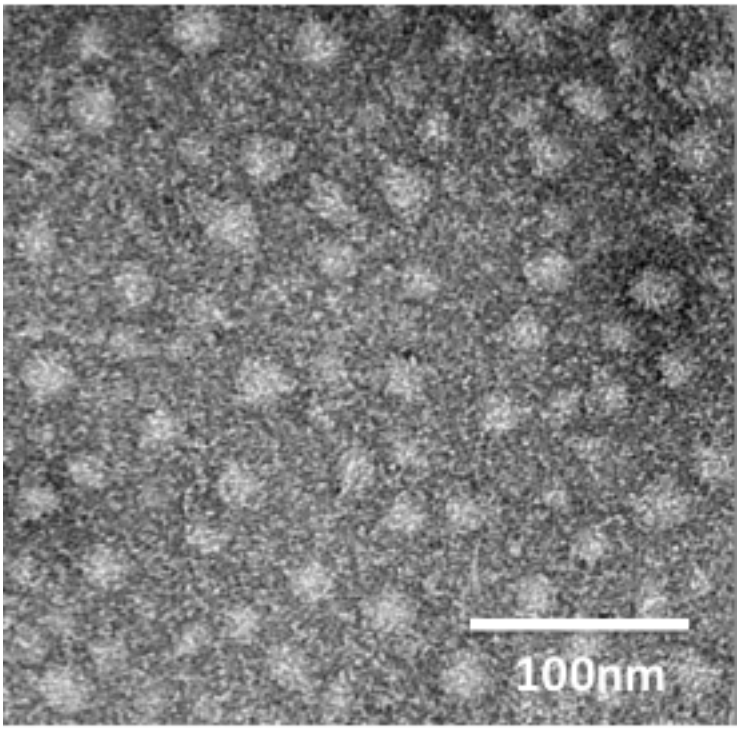


SI Figure 2. TEM image of siRNA nanocapsules (Scale bar = 20nm)

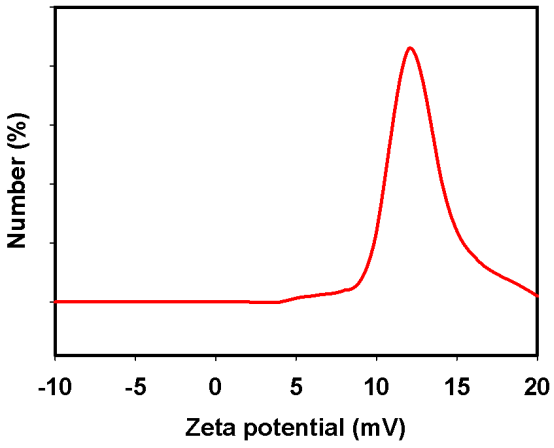


SI Figure 3. TEM image of BSA nanocapsules

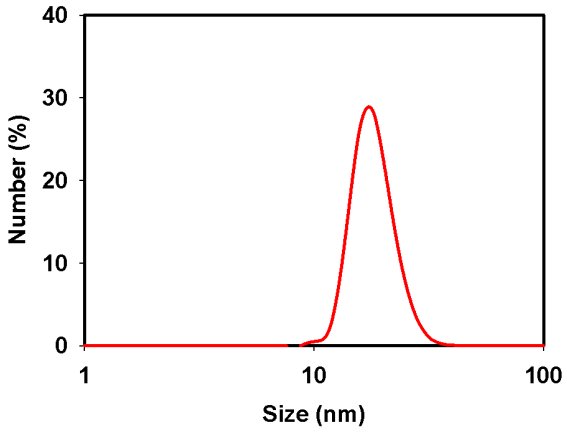
(TEM images of BSA nanocapsules were obtained on a Philips EM120 TEM at 100000X. Before observation, BSA nanocapsules were negatively stained using 1% pH 7.0 phosphotungstic acid (PTA) solution. The dark cores were not seen in the image.)



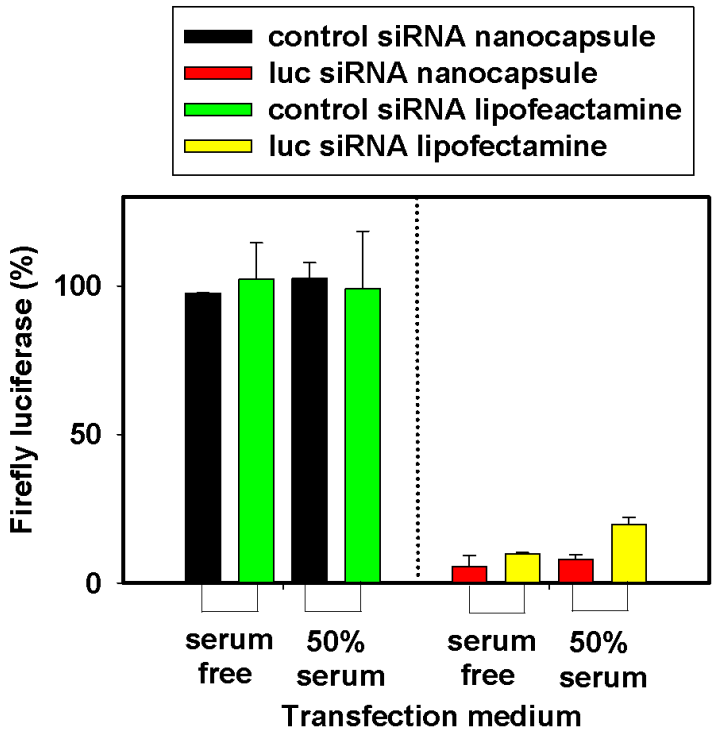
SI Figure 4. Surface charge of siRNA nanocapsules



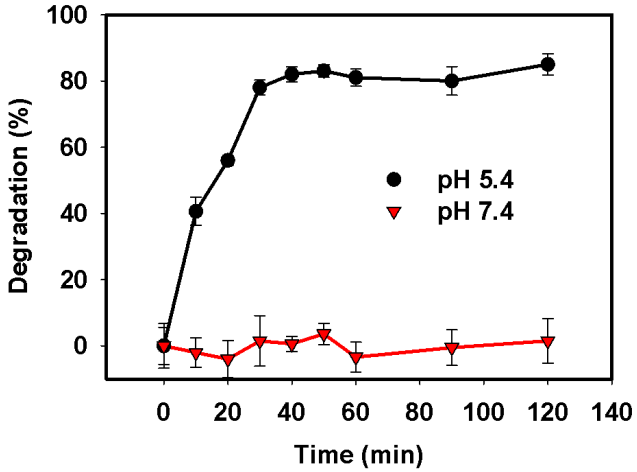
SI Figure 5. Size and size distribution of siRNA nanocapsules



SI Figure 6. Knockdown of luciferase in the presence of serum



SI Figure 7. The degradation of siRNA nanocapsules in 50 mM sodium acetate buffer (pH 5.4) and 50mM HEPES buffer (pH 7.4). The degradation (%) is calculated from the size of nanocapsules determined by dynamic light scattering.



SI Figure 8. Absorption spectra of siRNA (top) and siRNA-monomers complex (bottom) in 10mM HEPES buffer (pH=7.4).