Supporting information for:

Hierarchically-assembled Theranostic Nanostructures for siRNA Delivery and Imaging Applications

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[£] *This is a dedication for Dr. Michael J. Welch who passed away on May 6th, 2012.*

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S1. Preparation of cSCKs

The cSCKs were prepared in a two-step process; firstly amphiphilic block copolymer of $PAEA_{160}$-*b*-PS_{30} (5.0 mg) was directly dissolved in nanopure water (5.0 mL) and sonicated using a water bath sonicator for 10 min to obtain clear micellar solution at a concentration of 1 mg/mL. The solution was then allowed to stir overnight at room temperature. To the micellar solution (5.0 mL), sodium carbonate (20 μL of 1.0 M solution) was added to adjust the pH to *ca*. 8.0. A diacid crosslinker, 4,15-dioxo-8,11-dioxa-5,14-diazaoctadecane-1,18-dioic acid, (2.0 mg) was activated with HOBT (1.7 mg, 2.2 equiv. *per* COOH) and HBTU (4.8 mg, 2.2 equiv. *per* COOH) in 300 μL of DMF for 30 min. The activated crosslinker solution was slowly added to the micellar solution to crosslink *ca.* 5 % of the amines. The reaction mixture was allowed to stir overnight, and then transferred to a dialysis tubing (MWCO *ca.* 6000-8000 Da) and dialyzed against nanopure water for 3 d to obtain clear cSCK solution at 0.88 mg/mL. $(D_h)_{number}$ (DLS) = 21 ± 6 nm; $(D_h)_{volume}$ (DLS) = 48 ± 22 nm; $(D_h)_{intensity}$ (DLS) = 101 ± 59 nm. D_{av} (TEM) = 15 ± 3 nm. Zeta potential = 35 ± 2 mV (nanopure water, pH 5.5).

S2. Preparation of SCRs

Anionic cylinders were prepared by aqueous self assembly of amphiphilic block copolymer of PAA_{140}-*b*-P*p*HS_{50}, as reported previously.[1] To a 100 mL round bottom flask, equipped with a magnetic stir bar, PAA_{140}-*b*-P*p*HS_{50} (25 mg) was added followed by nanopure water (25 mL) to achieve a polymer concentration of 1 mg/mL. The mixture was allowed to stir at room temperature for 2 h followed by addition of nanopure water (60 mL) to achieve a final polymer concentration of *ca* 0.3 mg/mL. The solution was then allowed to stir at room

temperature. The following day, cylindrical micelles were allowed to undergo crosslinking of approximately 10% of the acrylic acid segments *via* amidation. To a 50 mL round bottom flask equipped with a magnet stir bar, a solution of cylindrical micelles (25 mL) was added. To this solution, a diamine crosslinker 2, 2'-ethylenedioxy)bis(ethylamine) (1 mg, 1.1 equiv. *per* COOH) was added from a stock solution in nanopure water and the reaction mixture was allowed to stir. After 30 min, 1-ethyl-3-(3-dimethylaminopropyl carbodiimide methiodide) (2.4 mg, 1.3 equiv. per COOH) was slowly added over a period of 2 min from a stock solution in nanopure water. The reaction mixture was allowed to stir overnight, and then transferred to a dialysis tubing (MWCO *ca.* 6000-8000 Da) and dialyzed against nanopure water for 3 d to obtain SCR solution at 0.28 mg/mL. Zeta potential = -30 ± 2 mV (nanopure water, pH 5.5).

S3. Preparation of hierarchical assemblies

Hierarchical assemblies were prepared by mixing cSCKs (600 μL) with SCRs (150 μL) to achieve an amine: carboxylate (N/C) ratio of 10:1. Zeta potential = 25 ± 2 mV (nanopure water, pH 5.5).

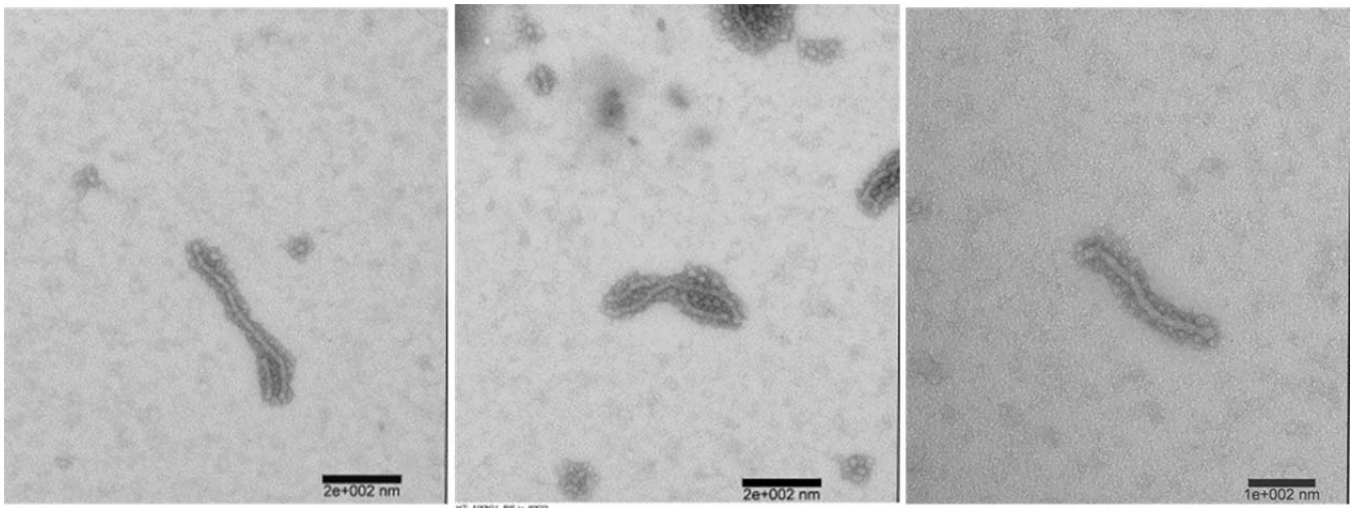

Figure S1. TEM micrographs of hierarchically-assembled nanoparticles

S4. Gel shift assays

Agarose gels (1 *wt*%) were prepared in Tris-acetate-EDTA buffer (Bio-Rad Laboratories, Inc., Hercules, CA). The siRNA (5'-Cy3-(sense strand)-GGCCACAUCGGAUUUCACU, M_w = 13814 g/mol, Dharmacon, Chicago, IL), either free or complexed to cylinders, Lipofectamine, the cSCKs or the hierarchal assemblies at nitrogen (total amine concentrations of the primary amines in the cSCKs and HAT)-to-phosphate (N/P) ratios ranging from 0.5-to-5 (1 μg siRNA/35 μL/well), were mixed with glycerol (50% *v*/*v*) prior to the electrophoresis. The weight and optimal (as set by the manufacturer) ratios were utilized for the cylindrical nanoparticles and Lipofectamine, respectively. Gel electrophoresis was carried out using a horizontal apparatus at 100 V for 20 min and fluorescence imaging of the separated siRNA bands was performed using a ChemiDoc XRS imager (Bio-Rad Laboratories, Inc.). The analysis and quantitative measurements of the siRNA bands were carried out by using Image Lab software (Bio-Rad Laboratories, Inc.).

S5. Laser scanning confocal microscopy (LSCM)

Human ovarian adenocarcinoma (OVCAR-3, 1 x 10^5 cells/well) cells were plated in glass-bottom six-well plate (MatTek Co., Ashland, MA) in DMEM medium. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h to adhere. Then, the medium was replaced with a fresh media 1-h prior to the addition of siRNA mixed with cylinders, cSCKs, hierarchical assemblies or Lipofectamine (200 nM final concentration of the 5'-Cy3-siRNA). The cells were incubated with the formulations for 3 h and washed extensively with PBS. Then, DRAQ-5 (Biostatus Ltd., Shepshed, Leicestershire, UK) was utilized to stain the nucleus (30 min incubation, followed by extensive washing with PBS). Cells were then fixed with 1% formaldehyde for 20 min, washed once with PBS. The cells were then stored in 1 mL PBS in the refrigerator and analyzed by laser scanning confocal microscopy (LSM 510, Zeiss, Jena, Germany). The images were collected under the same conditions (laser power, detector gain, *etc.*) for consistency, and $\lambda_{excitation}$ of 543 and 633 nm were utilized for the Cy3 and DRAQ-5, respectively.

S6. Death-siRNA transfection assays

RAW 264.7 mouse macrophages (2 x 10^4 cells/well) and OVCAR-3 cells (5 x 10^3 cells/well) were plated in 96-well plate in DMEM and RPMI-1640 medium (10% and 20% fetal bovine serum, for the RAW 264.7 and OVCAR-3, respectively and 1% penicillin/streptomycin). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h to adhere. Then, the medium was replaced with a fresh medium 1-h prior to the addition of the siRNA mixed with cylinders, Lipofectamine, cSCKs or hierarchical assemblies (100 nM final concentrations of AllStars death- or negative control-siRNA (Qiagen, Valencia, CA)) at N/P

ratio of 5. The cells were incubated with the various formulations for 24 h and washed extensively with PBS and the cell viability was measured 24 h later by measuring the relative cell viability of the cells treated with death-siRNA to the negative control-loaded nanoparticles. The Lipofectamine-siRNA complexes were prepared according to the manufacturer instructions and the transfection efficiency was measured following the same procedures of the siRNA-complexes.

S7. Radiolabeling of nanostructures

Bromine-76 ($t_{1/2}$ = 16.2 h, β+ = 57%, EC = 43%) was produced on the Washington University Medical School CS-15 cyclotron by the ^{76}Se (p,n) ^{76}Br nuclear reaction.[2] Hierarchically-assembled HAT nanoparticles, cSCKs, and SCRs (20 μg) were each incubated with 130 μCi ^{76}Br in 1x PBS buffer with the addition of bromoperoxidase (BPO, 0.6 units) in hydrogen peroxide (2.65 pmol) for 1 h at 0 °C. The radiochemical purity of ^{76}Br-radiolabeled nanoparticles was monitored by radio instant thin layer chromatography (radio-ITLC, Bioscan, Washington, DC). Additionally, chloramines-T method was also employed for ^{76}Br radiolabeling following a previously published procedure.[3]

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