Supplementary material for

Functional MnO Nanoclusters for Efficient siRNA Delivery

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S.1 Preparation of MnO nanoparticles (MONPs)

The 25 nm MONPs were synthesized by following a previously published protocol.[1-2] Mn-oleate (1.24 g) was added into 10 mL of 1-octadecene (90 %, Aldrich) to yield a transparent red solution. The mixture was heated to 160 °C and remained at this temperature under Ar protection for 1 h to remove water and oxygen and then heated to 300 °C with vigorous stirring. The reaction system was maintained at 300 °C for 1 h and then cooled down to room temperature. 20 mL of hexane was added into the product, followed by the addition of 80 mL acetone to precipitate the nanoparticles. The precipitate was collected by centrifugation and washed for 2 more times to remove excess surfactant and solvent.

To synthesize Mn-oleate complex, 1.98 g manganese chloride tetrahydrate ($MnCl_2$•$4H_2O$, 10 mmol, Sigma-Aldrich, 98%) and 6.09 g sodium oleate (20 mmol, TCI, 95%) were added to a mixture composed of 10 mL of ethanol, 40 mL of distilled water and 50 mL of n-hexane. The resulting mixture was heated to 70 °C and stirred overnight. The final product was washed several times by using water, and then heated to 50 °C to remove hexane.

S.2 Preparation of Alkyl-PEI2k-MnO/siRNA complexes

Alkyl-PEI2k was prepared by following our previously reported protocol.[3-5] Briefly,

branched PEI2k (Alfa Aesar) was reacted with 1-iododododecane (Aldrich) in ethanol. The purified product was obtained after lyophilization and was confirmed by ^{1}H NMR ($CDCl_3$).

Alkyl-PEI2k-MnOs were prepared by following the protocol below. Typically, 5 mg MnO nanoparticles (MONPs) in hexane were dried under argon and redispersed in 1 mL chloroform together with 10 mg Alkyl-PEI2k. Then, the mixed solution was slowly added into 1 mL water with sonication. The mixture was under shaking for overnight and the remaining chloroform was removed by rotary evaporation, which was then dialysed for 48 h to remove the free Alkyl-PEI2k. Alkyl-PEI2k-MnO/siRNA complexes at different N/P ratios (N/P ratios of 1:1, 2:1, 5:1, 10:1 and 20:1) were prepared by adding appropriate amount of Alkyl-PEI2k-MnOs into firefly luciferase siRNA (Ambion) (6 pmol) in PBS under agitation. The resulting mixture was incubated at room temperature for 20 min.

S.3 Agarose Gel Electrophoresis of Alkyl-PEI2k-MnO/siRNA Complexes

Alkyl-PEI2k-MnO/siRNA complexes were analyzed by 2% agarose gel electrophoresis and heparin decomplexation assay.[6] The gels were prepared with 2% agarose in Tris-acetate-EDTA buffer containing 0.5 μg/mL ethidium bromide. Gel electrophoresis was carried out at 100 V for 15 min and the gel was subsequently imaged using a LAS-3000 gel documentation system (Fujifilm Life Science, Japan). For heparin decomplexation assay, Alkyl-PEI2k-MnOs were first complexed with siRNA (N/P ratio = 20:1) at room temperature for 20 min. Then, various amounts of heparin (heparin/siRNA weight ratio 0, 1, 5, 20 and 100) were added and the mixtures were further incubated for 15 min. The samples were loaded onto a 2 % agarose gel again and subjected to electrophoresis as described above.

S.4 Cell Culture

The 4T1 breast cancer cells were purchased from the American Type Culture Collection (ATCC). To establish 4T1 cell line that stably expresses luciferase gene (4T1-fluc), transfection was done with pcDNA 3.1 cytomegalovirus-firefly luciferase (fluc) DNA and Lipofectamine 2000 (Invitrogen) using 80 % confluent 4T1 cells.[5] The cells were incubated for 48 h before the culture medium was changed. Selection was made by adding selective medium containing 1 mg/mL G418

antibiotic (Mediatech, Inc.) every 2-3 days. Two weeks later, the cells were analyzed and subcloned in 24-well plates. When reaching 80% confluence, the cells in the plate were imaged using a Xenogen IVIS-100 system (Caliper Life Sciences) after addition of the substrate D-luciferin (20 μL per well of 3 mg/mL stock). The 4T1-fluc clone with the highest fluc activity was selected for further studies. No significant difference between 4T1 and 4T1-fluc cells was observed in terms of proliferation or tumorigenicity. The cells were used when they reached 80 % confluence.

S.5 Fluorescence microscope and Confocal Microscopic Imaging Study

Fluc-4T1 cells were grown 50-60 % confluent on 4 well chambered LabTek II coverglass, treated with free Cy3-siRNA, Alkyl-PEI2k-MnO/Cy3-siRNA complexes, Lipofectmin2000/Cy3-siRNA as described above and, incubated for 3 h. Cells were next washed with PBS and replaced with fresh media. Fluorescence images of Alkyl-PEI2k-MnO/Cy3-siRNA complexes, free siRNA, Lipofectamine2000 as controls were carried out using a fluorescence microscope (Olympus IX81).

The CLSM images of the Alkyl-PEI2k-MnO/Cy3-siRNA complexes and Lipofectamine2000 groups were carried out using an inverted Zeiss LSM 700 confocal microscope equipped with CO_2 module, heating unit and heating plate using a 40x/0.75 M27 EC Plan-Neofluar objective. Imaging was carried out at 37 °C in 5% CO_2 with cells plated in chambered LabTek II coverglass. Images were acquired and processed with the Zeiss Zen 2009 image software and ImageJ (NIH). The fluorescence data shown are representative of at least three independent experiments.

S.6 In Vitro Transfection of Alkyl-PEI2k-MnO/siRNA Complexes

siRNA (6 pmol per well) and appropriate amount of Alkyl-PEI2k-MnOs were both diluted to 25 μL with fresh medium and were mixed at room temperature for 5 min; after that, the solution was incubated at room temperature for another 20 min. In the meantime, the cells in 96-well plate were washed with PBS and replaced with fresh medium (50 μL, each well). The transfection complexes were then added into the wells and the cells were incubated at 37 °C, 5% CO_2, 95-100% humidity for the indicated time period. Cells for positive controls were transfected with Lipofectamine using the recommended protocol from the manufacturer (Invitrogen). The luciferase

expression of fluc-4T1 cells was visualized 48 h post-transfection as described above. After transfection, MTT assay was conducted late by following a standard MTT assay protocol.

S.7 MRI Study of Transfected Cells

Fluc-4T1 cells were conducted with Alkyl-PEI2k-MnO/siRNA complexes at a N/P ratio of 10. After transfection for 3 h, cells were washed three times with PBS, harvested, and processed for MR imaging as described below. After carefully counted, cells (10^6, 2×10^6, 5×10^6, 10^7) were dispersed in a final concentration of 5% gelatin phantom (100 μL) crosslinked with glutaraldehyde inside microcentrifuge tubes.[7] T_1 weight images were acquired on a 7.05 Tesla small-animal MR scanner (Bruker Biospin). The following parameters were adopted in data acquisition: TR = 250 ms, TE = 4.5 ms, Flip Angle= 45°, thickness 0.5 mm, FOV 3 × 3 cm, NEX 4.0, Echo 1/1. The signal intensity of transfected cells was displayed as percentages relative to untreated cells and the T_1 weighted imaging was generated by NIH Image J (Bethesda, MD).

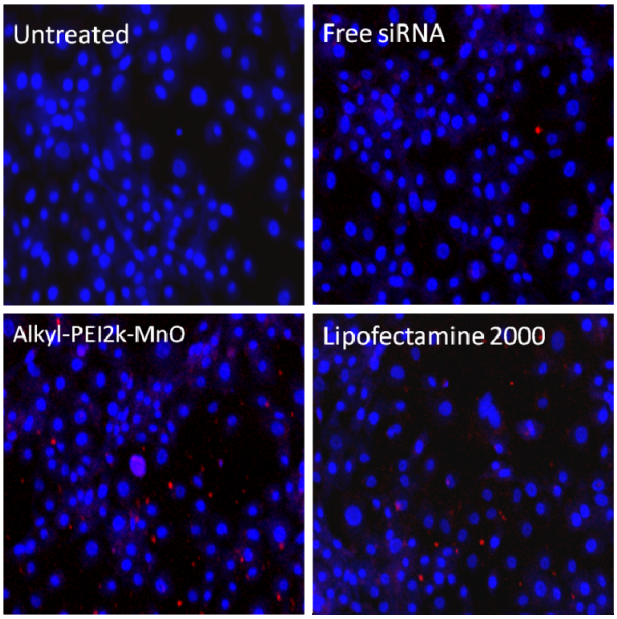

Fig. S1 Fluorescence microscopic images of 4T1-fluc cells after being treated with free Cy3-siRNA, Alkyl-PEI2k-MnOs/Cy3-siRNA, and Lipofectamine™ 2000/Cy3-siRNA. The untreated group was used as a control. Cell nuclei were also stained with DAPI.

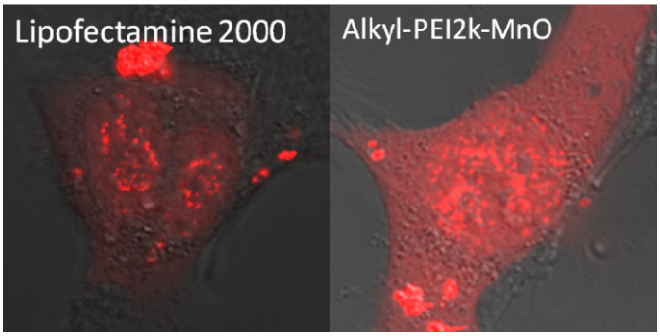

Fig. S2 Confocal microscopic images of 4T1-fluc cells conducted with Alkyl-PEI2k-MnOs/Cy3-siRNA and Lipofectamine™ 2000/Cy3-siRNA.

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