Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

Multifunctional Fluorescent-Magnetic Polyethyleneimine Functionalized Fe_3O_4/Mesoporous Silica Yolk/Shell Nanocapsules for siRNA Delivery

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Experimental section

Reagents: Fe_3O_4 NPs protected by oleylamine (OMA) and oleic acid (OA) were obtained as a gift from Ocean Nano Tech. 3-aminopropyltrimethoxysilane (APTMS, 95 %), cetyltrimethylammonium bromide (CTAB), Rhodamine B isothiocyanate (RITC), tetraethyl orthosilicate (TEOS≥ 98 %), polyethyleneimine (PEI, branched, Mw ~25000) were purchased from Sigma-Aldrich. The synthetic fluorescence labeled FAM-siRNA (sense strand, 5'-UUCUCCGAACGUGUCACGUTT-3', antisense strand, 5'-ACGUGACACGUUCGGAGAATT-3') and sequence specific siRNA for human β-actin (sense strand, UGAAGAUCAAGAUCAUUGCdTdT, antisense strand, GCAAUGAUCUUGAUCUUCAdTdT) were purchased from GenePharma Co. Ltd. (Shanghai, China). Diethypyrocarbonate (DEPC) and Lipofectamine 2000 were obtained from Invitrogen Life Science. All other chemicals were of analytical grade.

Characterization: TEM was performed on a JEOL-2100F transmission electron microscope under 120 kV accelerating voltage. Zeta potential measurements were determined by Dynamic Light Scattering (Zetasizer Nano ZS). Fluorescence spectra were performed with Eclipse fluorescence spectrophotometer (Varian, USA). The magnetic measurement was carried out by using a super-conducting quantum interference device magnetometer (SQUIDMPMS XL-7) with fields up to 1.5 T. Confocal laser scanning microscopy (CLSM) was operated on Olympus Fluoview FV1000. The nitrogen content was determined by elemental analysis (Perkin-Elmer 2400 II)

Preparation of Fe_3O_4@fmSiO_2 core-shell NPs: RITC was incorporated in the

silica coating on the Fe_3O_4 NPs surface by first making a RITC/APTMS/ethanol solution. In a typical procedure, 10 mg of RITC was covalently linked to 44 µL of APTMS in 0.75 mL ethanol under dark conditions for 2 days. The prepared RITC-APTMS stock solution was kept at 4 ºC [1]. Briefly, 1 mL of 10 mg mL^{-1} Fe_3O_4 NPs in chloroform was mixed with 20 mL of 0.2 M CTAB solution. After stirred vigorously for 30 min, the resulting solution was heated up to 60 ºC to evaporate the chloroform. 20 mL of pure water and 5 µL of the RITC-APTMS solution were added into the as-prepared CTAB-stabilized Fe_3O_4 NPs. Then, 0.1 M NaOH was added into the mixture to adjust the pH value to ~ 8. Then, 1 mL of 20 % TEOS in ethanol was injected at a 30 min intervals. The reaction mixture reacted for 24 h under gentle stirring. The obtained Fe_3O_4@fmSiO_2 core-shell NPs were centrifuged and rinsed with ethanol repeatedly to remove the excess precursors and CTAB molecules and then dispersed in 10 mL of ethanol.

Preparation of PEI coated Fe_3O_4@fmSiO_2 yolk-shell NCs: To perform PEI coating, 1 mL of as-prepared Fe_3O_4@fmSiO_2 core-shell NPs were mixed with 1 mL of 10 mg mL^{-1} PEI in absolute ethanol for 60 min. The obtained PEI-Fe_3O_4@fmSiO_2 core-shell NPs were washed with ethanol and water for several times and finally dispersed in water. Then, the solution was heated at 50 ºC for 50 min under stirring to obtain PEI-Fe_3O_4@fmSiO_2 yolk-shell NCs. Here, water was used as a mild etching agent to form a yolk-shell nanostructure. The as-prepared PEI-Fe_3O_4@fmSiO_2 yolk-shell NCs were washed and dispersed in diethypyrocarbonate (DEPC) water for further use.

Cell culture: A human Hela cancer cell lines were grown as a monolayer in a humidified incubator at 37 °C in a 95 % air/5 % CO_2 in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10 % fetal bovine serum (FBS).

Preparation of siRNA/PEI-Fe_3O_4@fmSiO_2 yolk-shell NCs nanocomplex: 40 pmol of siRNA was mixed with 7-42 μg of PEI-Fe_3O_4@fmSiO_2 yolk-shell NCs in aqueous solution to obtain NCs/nuclei acid (N/P) ratio of 4-22. After incubating 15 min, the electrophoretic mobility of the mixture was visualized on a 2 % (w/v) agarose gel containing ethidium bromide. The measurement was carried out for 30 min at 90 V in TAE buffer and captured using a gel imaging system (UV Itec, BTS-20-M). A commercial available cationic liposomal transfection agent， Lipofectamine 2000, was used as a positive control.

Cell uptake: Hela cells were seeded on coverslips in a 24-well plate at a seeding density of 2.5×10^4 cells/well. After 24 h, the old medium was discarded. Then, fresh culture without serum and antibiotics were added in the cells. The transfection procedure of Lipofectamine 2000 was performed according to the manufacturer’s protocol. Briefly, 40 pmol siRNA diluted in 50 μL opti-MEM was mixed with 1 μl Lipofectamine 2000 diluted in 50 μL opti-MEM, respectively. The solutions were incubated for 20 min at room temperature, and then added into the plates. To prepare siRNA-Fe_3O_4@fmSiO_2, PEI-Fe_3O_4@fmSiO_2 yolk-shell NCs (28 μg and 42 μg) were mixed with siRNA (40 pmol) in RNA-free eppendorf tube for 15 min to form siRNA-Fe_3O_4@fmSiO_2 (N/P = 15 and 22), respectively. Free FAM-siRNA, FAM-siRNA-Lipofectamine 2000, FAM-siRNA-Fe_3O_4@fmSiO_2 (N/P = 15 and 22)

and FAM-siRNA-Fe_3O_4@fmSiO_2 (N/P = 22) under an external magnetic field were gently dripped into wells respectively. The final medium volume was 1ml and the final concentration of siRNA in each well is 40 nM. After incubated for 1 h, cells were washed and treated with Hoechst 33342 (10 μg mL^{-1}) for 15 min to stain the nuclei for fluorescence imaging. To investigate the property of siRNA release, Hela cells were treated with siRNA-Fe_3O_4@fmSiO_2 (N/P = 22) for 1 h, and then incubated for another 4 h with fresh medium. The mean fluorescent intensity of Hela cells uptake of FAM-siRNA (green) and Fe_3O_4@fmSiO_2 (red) in Hela cells was measured by CLSM (Olympus Fluoview FV1000). Three images of each group were selected to measure the mean intensity.

Flow cytometer analysis was actualized to assess the ability of Fe_3O_4@fmSiO_2 yolk-shell NCs to transfer siRNA into cells. Hela cells were seeded at 5×10^4 cells/well in 6-well plates and left overnight to adhere. The medium was changed to both antibiotics and FBS free medium. Then, FAM-siRNA-Lipofectamine 2000, FAM-siRNA-Fe_3O_4@fmSiO_2 and FAM-siRNA-Fe_3O_4@fmSiO_2 under an external magnetic field were added into the cells respectively. After incubation for 24 h, the cells were washed with PBS, and the uptake of FAM-siRNA were detected by a Coulter Epics XL•MCL flow cytometer (Beckman Coulter).

Cytotoxicity Assessment: The cellular toxicity was performed using a standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Hela cells were plated in 96-well plates (5,000 cells per well). After 1 day, Lipofectamine 2000, PEI-Fe_3O_4@fmSiO_2 yolk-shell NCs, siRNA-Lipofectamine 2000 and

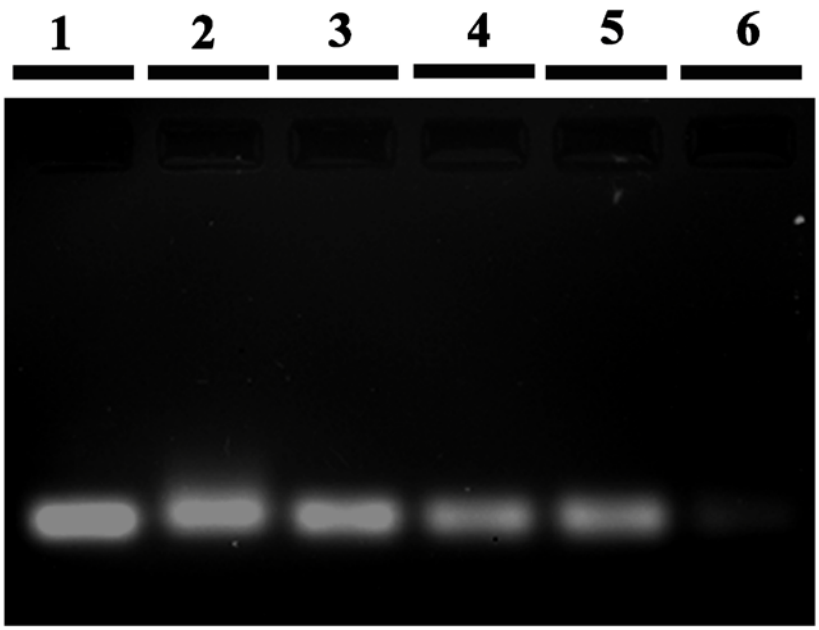
siRNA-Fe_3O_4@fmSiO_2 (N/P = 15 and 22) treated with and without an external magnetic fieldin opti-MEM medium were replaced the original medium and cultured for 12 h at 37 °C, respectively. The final siRNA concentration is 40 nM. Then, medium were changed with fresh medium contain 10 % FBS. After incubation another 48 h, MTT stock solution (10 μL, 5 mg mL^{-1}) was added to each well and the mixture was incubated under cell culture for another 4 h. Every well was then added DMSO (150 μL) to dissolve the formation crystals. Meanwhile, the cytotoxity of different concentrations of PEI-Fe_3O_4@fmSiO_2 yolk-shell NCs and PEI-Fe_3O_4@fmSiO_2 yolk-shell NCs treated with an external magnetic field in Hela cells were determined with the same process. The absorbance of each sample was monitored with a microplate reader at a wavelength of 490 nm. Cell viability was determined by Equation (1):

$$\text{Cell Viability (\%)} = \text{Abs}_{\text{(test cells)}}/\text{Abs}_{\text{(reference cells)}} \times 100\ \% \qquad (1)$$

Western blotting analysis: Hela cells were seeded in 6-well plates (50,000 cells per well) one day before transfection. Following attachment, the medium was removed from the plate and replaced with fresh medium without FBS and antibiotic. The transfection solution was prepared by combining PEI-Fe_3O_4@fmSiO_2 yolk-shell NCs with β-actin siRNA at the N/P ratio of 22 for 15 min. Final siRNA concentration in the transfection solution was 40 nM. The medium was removed from the plate an replaced with the transfection solution. Then, PEI-Fe_3O_4@fmSiO_2 yolk-shell NCs, siRNA-Fe_3O_4@fmSiO_2 (N/P = 22), siRNA-Fe_3O_4@fmSiO_2 with an external magnetic field (N/P = 22) were added into the plates, respectively. The transfection procedure

of Lipofectamine 2000 was performed according to the manufacturer's protocol. The final siRNA concentration is 40 nM. After 12 h incubation with the trasfection solution, the old serum was changed to new serum with FBS for another 48 h. After that, the transfected cells were lysed at 4 °C and centrifuged. BCA kit was used to equal the amounts of protein. Protein were separated by gel electrophoresis on a 12 % polyacrylamide gel containing SDS and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5 % milk in Tris-buffered saline tween-20 (TBST) for 2 h. After being washed with TBST, the membranes were incubated with primary antibody β-actin (1:300) and GAPDH (1:1000) in 5 % milk/TBST overnight at 4 °C. After washing, the membranes were incubated with secondary antibody β-actin (1:300) and GAPDH (1:1000) for 2 h at room temperature. After washing three times for 10 min at room temperature in TBST, the signal was detected by ECL western blot detection kit (Beyotime Biotech, China). For quantifying image intensity, ImageJ software was used. Gene expression was calculated as a ratio of the band intensity of β-actin protein to that of GAPDH protein, and then the ratio of each sample was normalized to that of sample without treatment.

Statistical analysis: All experiments were performed in triplicate. The results are reported as mean ± standard deviation. Statistical significance was evaluated using two-tailed heteroscedastic Student's t-tests according to the TTEST function in Microsoft Excel. The difference between groups was considered statistically significant when the P-value was less than 0.05.



Lane 1	Control
Lane 2	siRNA-Lipofectamine 2000
Lane 3	N/P ratio = 4
Lane 4	N/P ratio = 7
Lane 5	N/P ratio = 15
Lane 6	N/P ratio = 22

Fig. S1. Agarose gel electrophoresis and β-actin decomplexation assay of free siRNA (Lane 1), siRNA-Lipofectamine 2000 (Lane 2), siRNA-Fe_3O_4@fmSiO_2 at various N/P ratios. The final siRNA concentration is 40 nM.

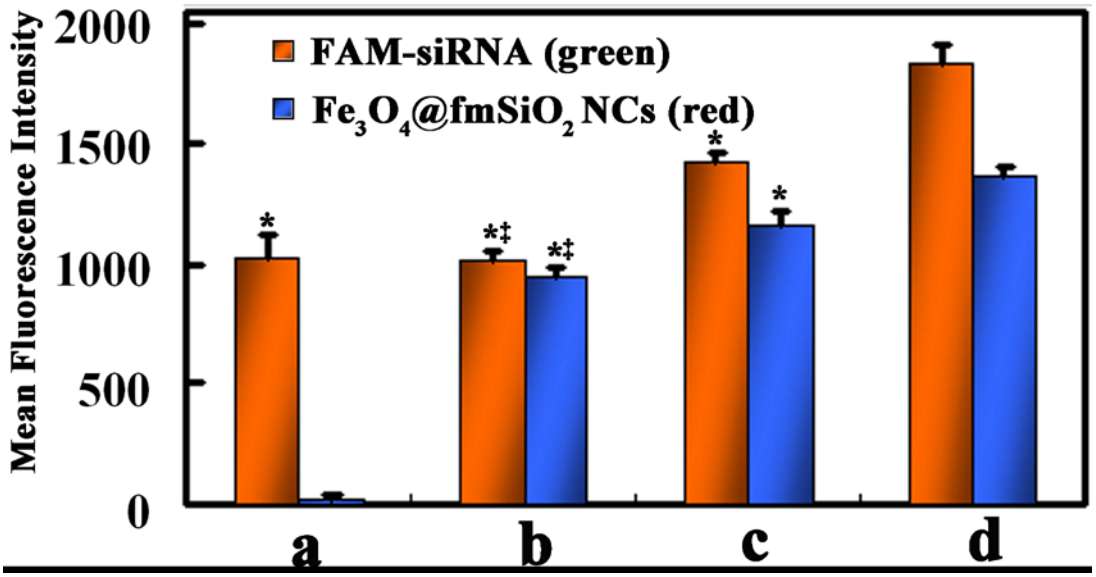


Fig. S2. Quantitative comparison of FAM-siRNA-Lipofectamine 2000 (a), FAM-siRNA-Fe_3O_4@fmSiO_2 at N/P ratio of 15 (b) FAM-siRNA-Fe_3O_4@fmSiO_2 at N/P ratio of 22 (c) and FAM-siRNA-Fe_3O_4@fmSiO_2 at N/P ratio of 22 treated with an external magnetic field (d) uptake in Hela cells by measuring the fluorescent intensity of FAM-siRNA (green) and Fe_3O_4@fmSiO_2 (red). The final siRNA concentration is 40 nM. Mean ± SD are shown. *$p < 0.05$ when compared with FAM-siRNA-Fe_3O_4@fmSiO_2 at N/P ratio of 22 treated with an external magnetic field. ‡$p < 0.05$ when compared with FAM-siRNA-Fe_3O_4@fmSiO_2 at N/P ratio of 22.

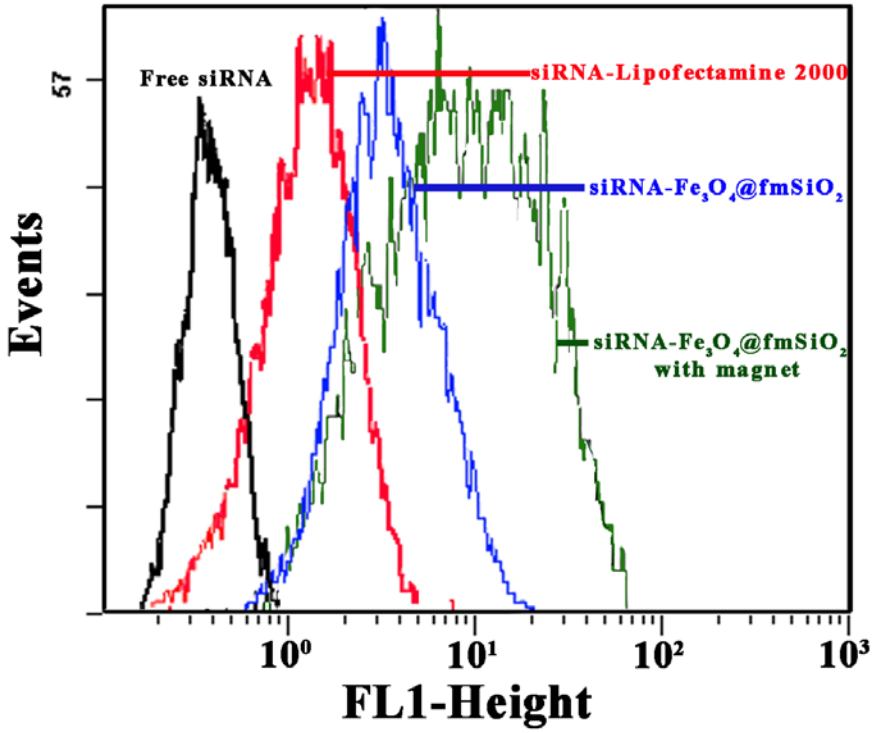


Fig. S3. Flow cytometric analysis of Hela cells transfected by (A) free FAM-siRNA, (B) FAM-siRNA-Lipofectamine 2000, (C) FAM-siRNA-Fe_3O_4@fmSiO_2 at N/P ratio of 22 (D) FAM-siRNA-Fe_3O_4@fmSiO_2 at N/P ratio of 22 in the presence of an external magnet field. The final siRNA concentration is 40 nM.

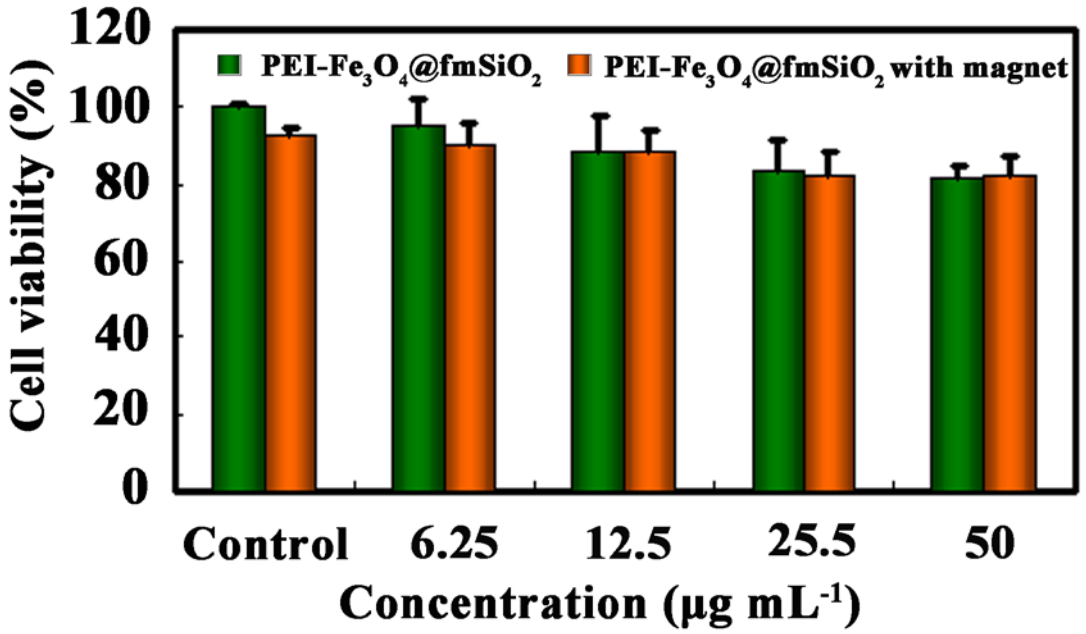


Fig. S4. Cytotoxicity of Hela cells incubated with different concentrations of PEI-Fe_3O_4@fmSiO_2 yolk-shell NCs and PEI-Fe_3O_4@fmSiO_2 yolk-shell NCs with an external magnetic field.

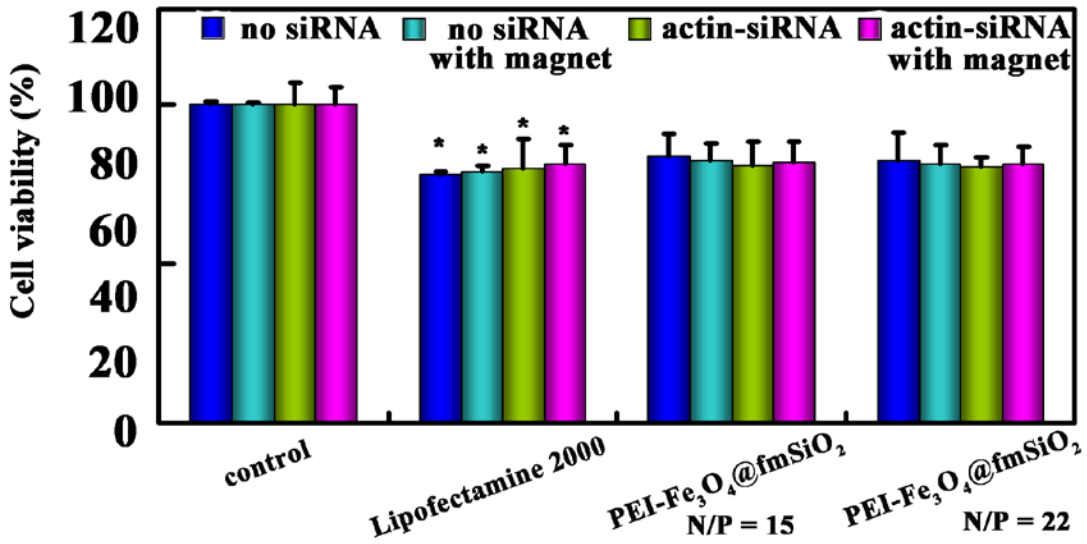


Fig. S5. Cytotoxicity of Lipofectamine 2000, PEI-Fe_3O_4@fmSiO_2 yolk-shell NCs, siRNA-Lipofectamine 2000 and siRNA-Fe_3O_4@fmSiO_2 (N/P = 15 and 22) treated with and without an external magnetic field in Hela cells, respectively. The final siRNA concentration is 40 nM. Mean ± SD are shown. *$p < 0.05$ when compared with control.

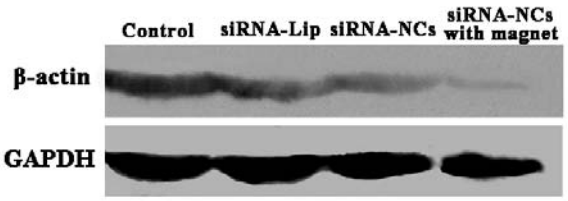


Fig. S6. Gel images of β-actin (target) and GAPDH (control) from western blot analysis. The β-actin protein level was effectively suppressed to 40 % and 54 % of Hela cells treated with siRNA-Lipofectamine 2000 and siRNA-Fe_3O_4@fmSiO_2 respectively. Compared with Hela cells incubated with siRNA-Fe_3O_4@fmSiO_2, siRNA-Fe_3O_4@fmSiO_2 under an external magnetic field induced much higher gene silencing (~ 23 %).

[1] J. Kim, H. S. Kim, N. Lee, T. Kim, H. Kim, T. Yu, I. C. Song, W. K. Moon and T. Hyeon, *Angew. Chem. Int. Ed.* 2008, **47**, 8438-8441.