Supporting Information

$Ce^{3/4+}$ Cation-Functionalized Maghemite Nanoparticles Towards siRNA-Mediated Gene Silencing

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A. Experimental procedure for nanocarrier fabrication - Starting neat neutral magnetite (Fe_3O_4) nanoparticles (Fe_3O_4 NPs, basic Massart hydrolytic method)

A solution of $FeCl_3$•$6H_2O$ (240.0 mg, 0.9 mmol) dissolved in deoxygenated milliQ purified H_2O (4.5 mL) was mixed with an aqueous solution of $FeCl_2$•$4H_2O$ (97.5 mg, 0.45 mmol, 4.5 mL H_2O). This solution was kept under N_2 and ultrasonicated (Bransonic® ultrasonic cleaner bath, 2510E MTH model, 42 KHz at full power) for 5-10 min at room temperature. Then, a concentrated 24% weight aqueous NH_4OH (0.75 mL) was introduced in one shot, resulting in an immediate black precipitation of magnetite (Fe_3O_4) NPs. Sonication was continued for 10 additional minutes. Resulting Fe_3O_4 NPs were transferred into a glass bottle (100 mL), magnetically decanted (using a strong external magnet), and washed with ddH_2O (3 x 40 mL) until neutrality. Then, brilliant black free flowing magnetite NPs were stored as a 30 mL NP suspension in ddH_2O before any further processing. Then, an ageing process must be at least executed for a minimum storage time in these conditions of 2h (room temperature).

B. Experimental procedure for DoE-optimized nanocarrier fabrication (MINITAB® 16 DoE software, version 16.2.4, Minitab Inc., *profile optimizer tool*) - CAN_{DOE}-stabilized maghemite nanoparticles (CAN_{DOE}-γ-Fe_2O_3 NPs)

First, the former aqueous magnetite NP suspension (30 mL, 4.22 h ageing time) was magnetically decanted to separate the magnetite NPs from its aqueous storage phase. Ceric ammonium Nitrate (CAN, $(NH_4)_2Ce(IV)(NO_3)_6$, 500.0 mg, 0.912 mmol) dissolved in 6.0 mL MeCOMe was introduced onto decanted magnetite NPs, followed by the addition of degassed milliQ purified H_2O (18.0 mL). The corresponding mixture was ultrasonicated using a high-power sonicator (Sonics®, Vibra cell, 750 Watt, power modulator set-up at 25%) equipped with a titanium horn (0.5 h, 0°C) under an inert argon atmosphere.

At this stage and optionally, the resulting highly stabilized hydrophilic CAN_{DOE}-γ-Fe_2O_3 NPs might be purified for other purposes [washing with ddH_2O (3 x 10 mL) using an Amicon® Ultra-15 centrifugal filter devices (100K) processed at 4,000 rpm during 5-6 min (rt, 18°C) and re-dispersed in ddH_2O (15 mL)] or alternatively processed for *b*-PEI (25.0 kDa branched PEI polymer) functionalization/decoration using mild aqueous contacting (corresponding experimental details mentioned below, section C).

NP cleaning/washing using an Amicon® Ultra-15 centrifugal filter device (100KDa)

Selected characterization data:

• Average NP TEM/DLS diameters: 6.61±2.04 and 60.00±1.98 nm (DLS, PDI: 0.136) respectively

• ζ potential: +45.7±1.88 mV

• w/w Ce/Fe ratio: 0.1000

• Average NP concentration: 5.8 mg/mL) before any further processing

• Kaiser test (free reactive primary amine quantification) - Experimental protocol for NP derivatization (coordination/contacting mode) & reactive primary amine quantification by UV spectroscopy.

To 1.0 mL of an aqueous suspension of CAN_{DOE}-γ-Fe_2O_3 NPs (5.8 mg/mL) was added 0.2 mL (2.58 mmol) of 1,4-diaminobutane (H_2N-$(CH_2)_4$-NH_2), and the reaction medium was gently shaken overnight at room temperature (20°C). At reaction completion, polyNH_2-modified CAN_{DOE}-γ-Fe_2O_3 NPs were cleaned before UV Kaiser test/ninhydrin reaction by three sequential centrifuge precipitation-ddH_2O washing steps (12,500 rpm, 5°C) for removal of the 1,4-diamine in excess.

Resulting polyNH_2-modified CAN_{DOE}-γ-Fe_2O_3 NPs were equally divided into two 13x100 mm-sized test tubes and freeze dried (lyophilization, -50°C, 0.06 mbar). Each weighed product was sequentially added with (i) 75.0 µL of an ethanol solution of phenol (PhOH, 40.0 g, 10 mL EtOH), (ii) 75.0 µL of an ethanol solution of ninhydrin (2.5 g dye, 14.0 mmol, 50.0 mL EtOH) and 100.0 µL of an aqueous KCN pyridine-containing solution (65.0 mg/1.0 mmol KCN dissolved in 100.0 mL ddH_2O = solution A - then 2.0 mL of solution A were diluted with 100 mL of pyridine). All the test tubes were then placed on a heating block pre-adjusted to 100°C for 10 min (ninhydrin reaction with accessible primary outer NH_2 groups). Then, each test tube was added with an aqueous 60% v/v solution of EtOH in ddH_2O (4.8 mL) followed by medium filtration (0.22 µm Millipore filter). A 0.5 mL aliquot of the filtered solution was then diluted with the former aqueous 60% v/v solution of EtOH in ddH_2O (4.5 mL). Finally, this diluted medium was UV assayed (UV spectrophotometer) at a λ_{max} of 570 nm (ninhydrin dye) to quantify the number of accessible reactive primary amine functional groups present onto the NP surface (mmol accessible NH_2 groups/g NPs). This test afforded a value of 0.272±0.023 μmol accessible NH_2 groups/mg CAN_{DOE}-γ-Fe_2O_3 NPs.

C. Experimental procedure for DoE-optimized nanocarrier fabrication – *b*-PEI-decorated CAN_{DOE}-γ-Fe_2O_3 NPs (*b*-PEI_{25}-CAN_{DOE}-γ-Fe_2O_3 NPs) - Optimal aqueous PEI contacting process/experimental protocol

The former corresponding CAN_{DOE}-γ-Fe_2O_3 NPs aqueous suspension (section B, 1.0 mL, Fe: 1.93 mg/mL – 1.93 mg total Fe, 0.0346 mmol Fe, ICP-AES measurement) was diluted to 25.0 mL using milliQ purified H_2O. Then, 10.13 mg of 25kDa branched *b*-PEI (0.4053 μmol, PEI/Fe Wt ratio: 5.25) were added at room temperature to the NP suspension as an aqueous solution (10.0 mg *b*-PEI/mL) and the medium was shaken overnight at room temperature (orbital shaker). At completion of such a mild *b*-PEI contacting/NP surface decoration, resulting crude *b*-PEI_{25}-CAN_{DOE}-γ-Fe_2O_3 NPs were washed 3 times (3 x 10 mL ddH_2O) using an Amicon® Ultra-15 centrifugal filter device (100K) operated at 4,000 rpm (5 min) followed by a size exclusion process performed using centrifugation (8,000 rpm/min, 16 min, 18°C and 7,000 rpm, 10 min, 18°C) to afford cleaned ***b*-PEI_{25}-CAN_{DOE}-γ-Fe_2O_3 NPs**.

Selected characterization data:

• Average NP TEM/DLS diameters: 6.86±1.55 nm and 82.90±1.26 nm (DLS, PDI: 0.195) respectively

• ζ potential: +31.1 mV

• w/w Ce/Fe ratio: 0.1000

• TGA weight loss (200-410°C temperature range): 73.62%

D. DoE-optimized fabrication of CAN-γ-Fe_2O_3 NPs – Complementary graphs & data relating to the fabrication of optimized CAN_{DOE}-γ-Fe_2O_3 NPs

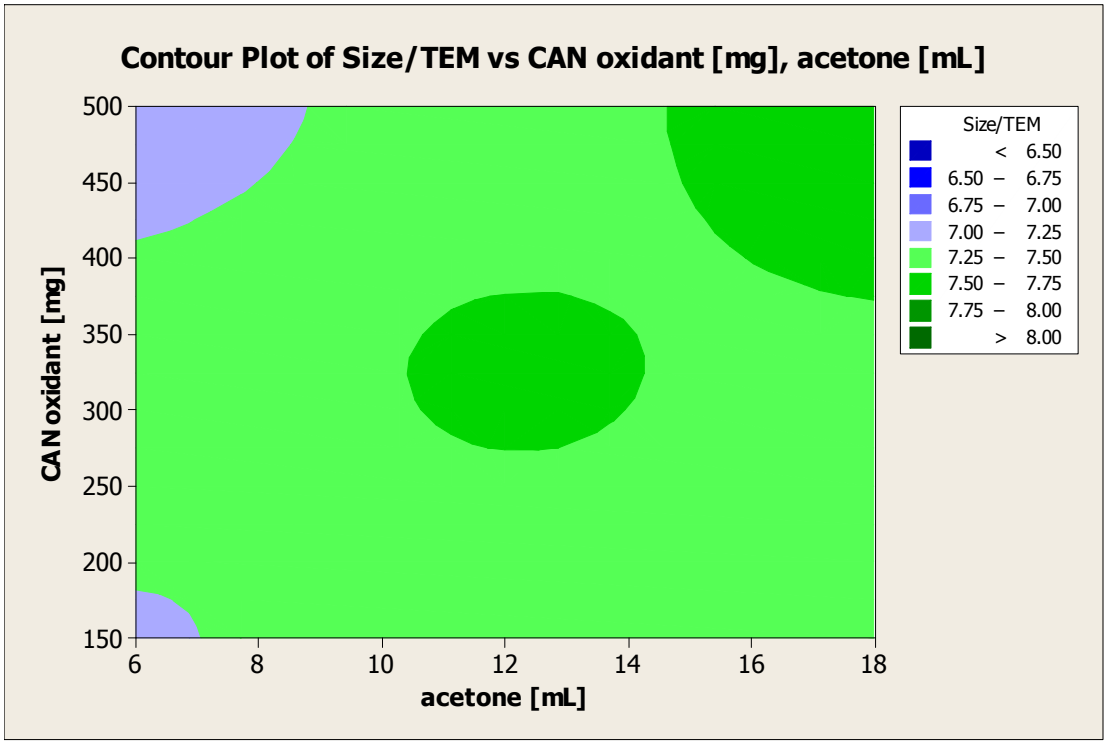


Figure SI-1. DoE-optimized fabrication of **CAN_{DOE}-γ-Fe_2O_3 NPs** - 2D Contour plot of **NP TEM sizes** *vs.* CAN oxidant (mg) & MeCOMe (mL) amounts

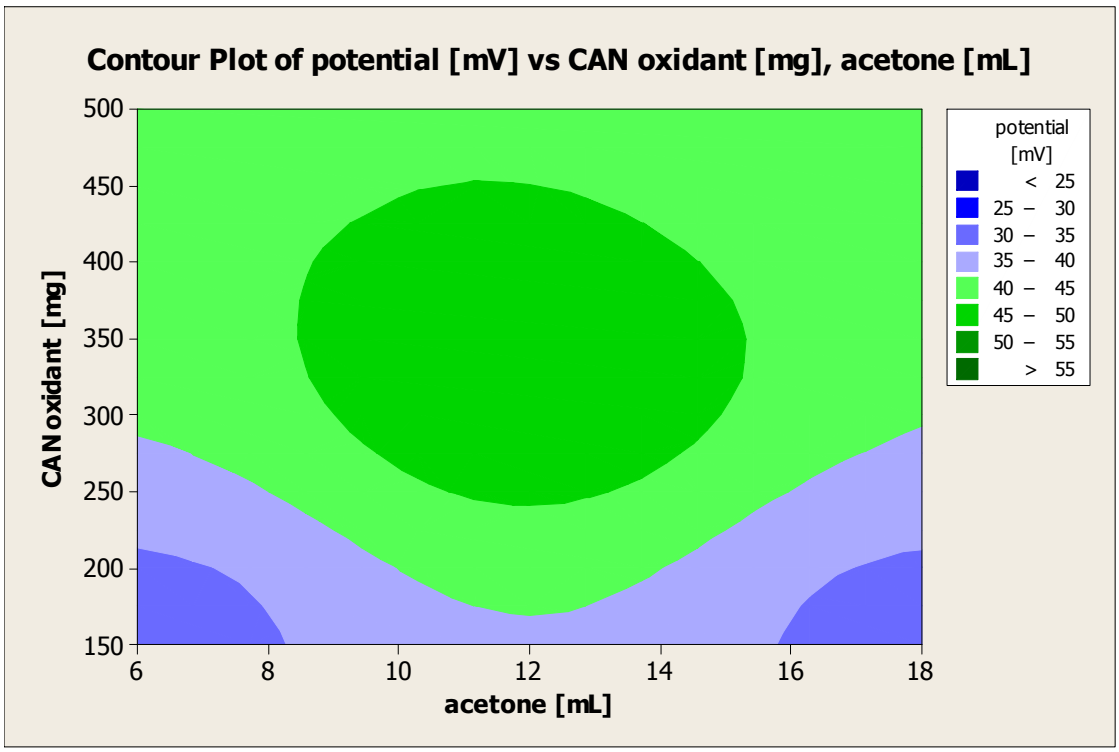


Figure SI-2. DoE-optimized fabrication of **CAN_{DOE}-γ-Fe_2O_3 NPs** - 2D Contour plot of **NP ζ potential** values *vs.* CAN oxidant (mg) & MeCOMe (mL) amounts

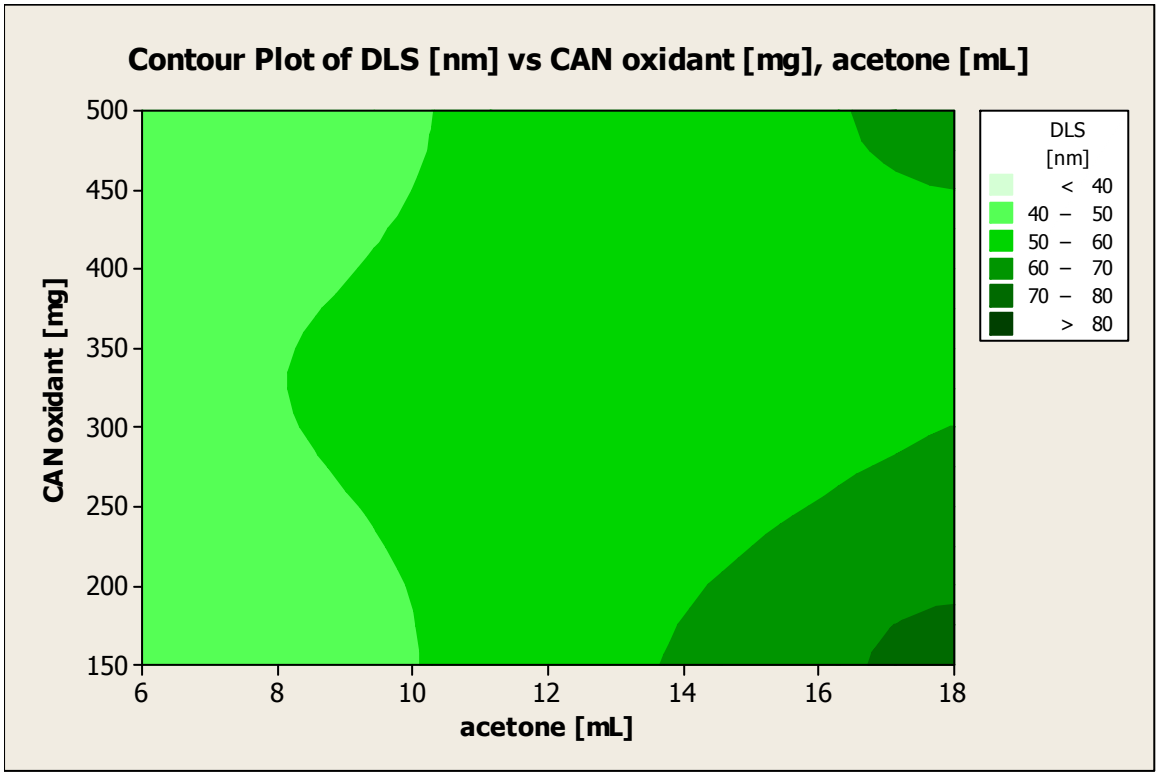


Figure SI-3. DoE-optimized fabrication of **CAN_{DOE}-γ-Fe_2O_3 NPs** - 2D Contour plot of **NP hydrodynamic size (DLS)** values *vs.* CAN oxidant (mg) & MeCOMe (mL) amounts

	Response	Goal	Lower	Target	Upper	Weight	Importance
C5	DLS [nm]	Minimize		40	50	1	1
C7	potential [mV]	Maximize	40	50		1	2
C10	Ce/Fe wt ratio	Maximize	0.05	0.1		1	4
C11	Size/TEM	Minimize		6.5	7.5	1	3

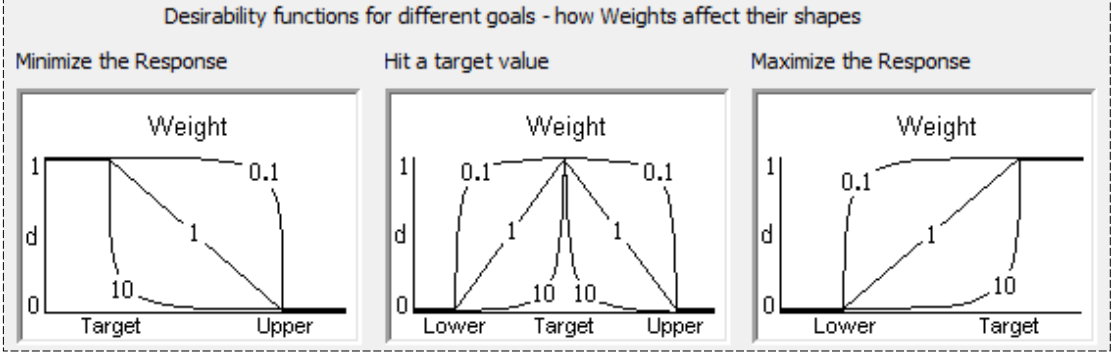


Figure SI-4. DoE-optimized fabrication of **CAN_{DOE}-γ-Fe_2O_3 NPs - NP specifications** for MINITAB® 16 software profile optimizer tool calculations

E. Optimized CAN_{DOE}-γ-Fe_2O_3 NPs – Additional characterization data

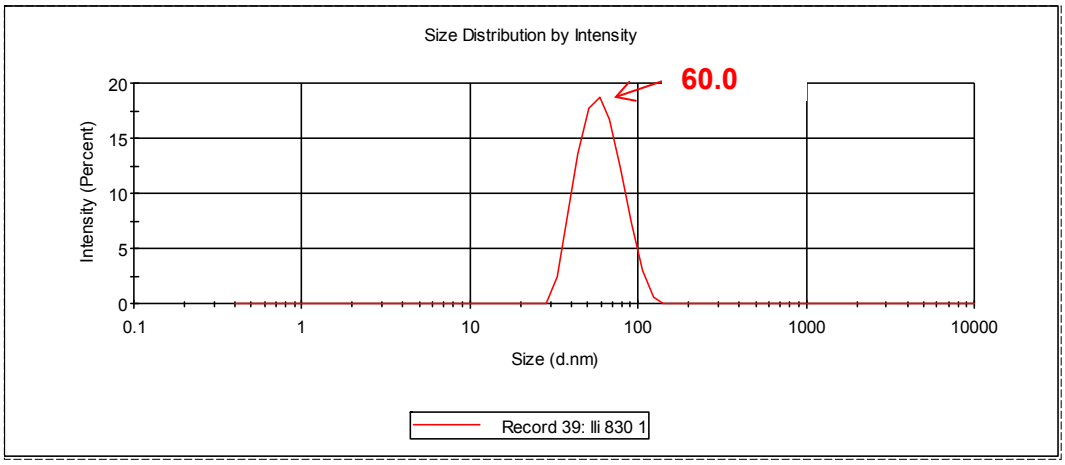


Figure SI-5. DLS analysis of DoE-optimized ultra-small 6.61±2.04 nm-sized core **CAN_{DOE}-γ-Fe_2O_3 NPs (**MINITAB® 16 software profile optimizer tool output)

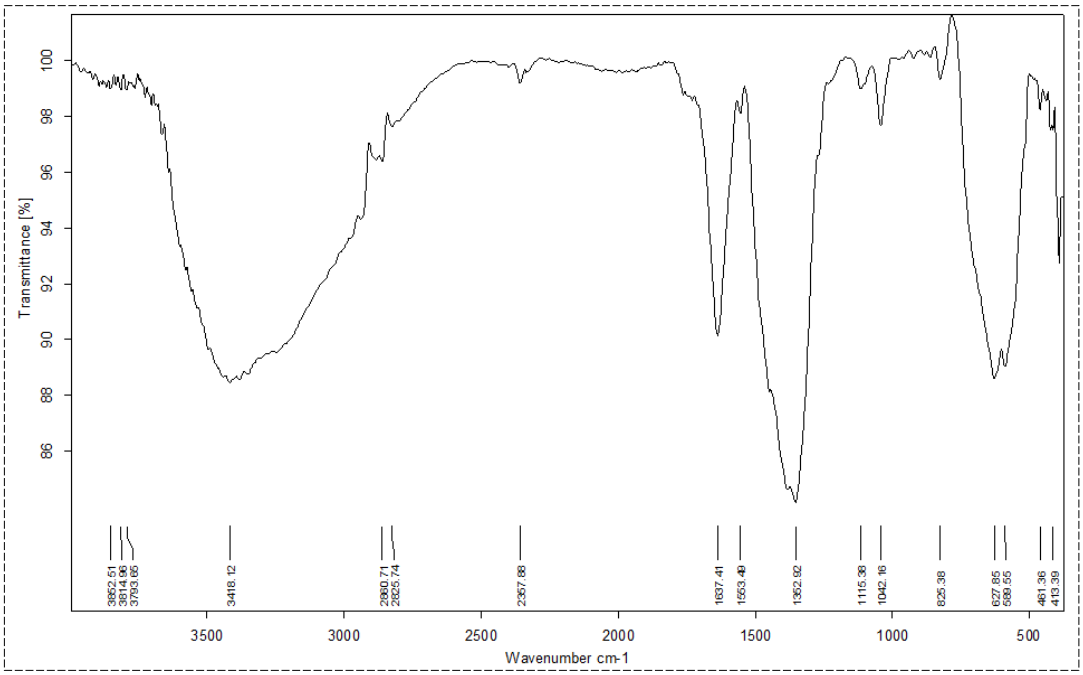


Figure SI-6. FT-IR spectrum of DoE-optimized ultra-small core 6.61±2.04 nm-sized **CAN_{DOE}-γ-Fe_2O_3 NPs (**MINITAB® 16 software profile optimizer tool output)

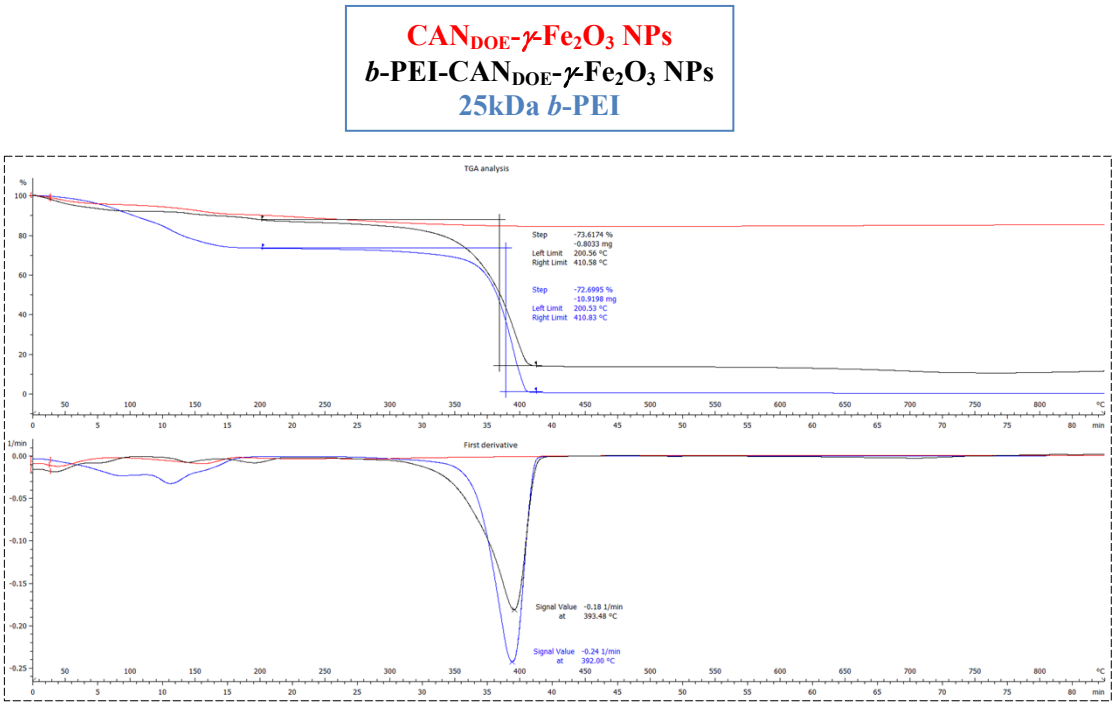


Figure SI-7. TGA thermogram (*top*)& weight loss derivative function (*bottom*) graphs of (i) DoE-optimized ultra-small 6.61±2.04 nm-sized core **CAN$_{DOE}$-γ-Fe$_2$O$_3$ NPs (**MINITAB® 16 software profile optimizer tool output, *red curves*), (ii) ***b*-PEI$_{25}$-CAN$_{DOE}$-γ-Fe$_2$O$_3$ NPs** (*black curves*), & (iii) pure **25kDa *b*-PEI** polymer (*blue curves*)

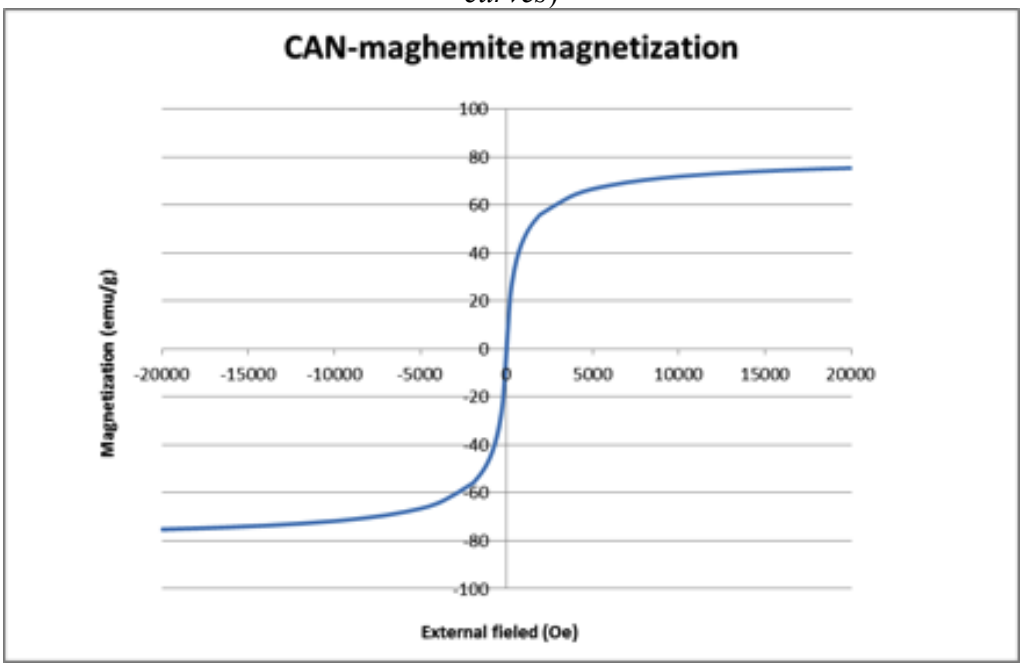


Figure SI-8. DoE-optimized ultra-small 6.61±2.04 nm-sized core **CAN$_{DOE}$-γ-Fe$_2$O$_3$ NPs (**MINITAB® 16 software profile optimizer tool output): SQUID magnetization profile (saturation magnetization *Ms*: 75.2 emu/g)

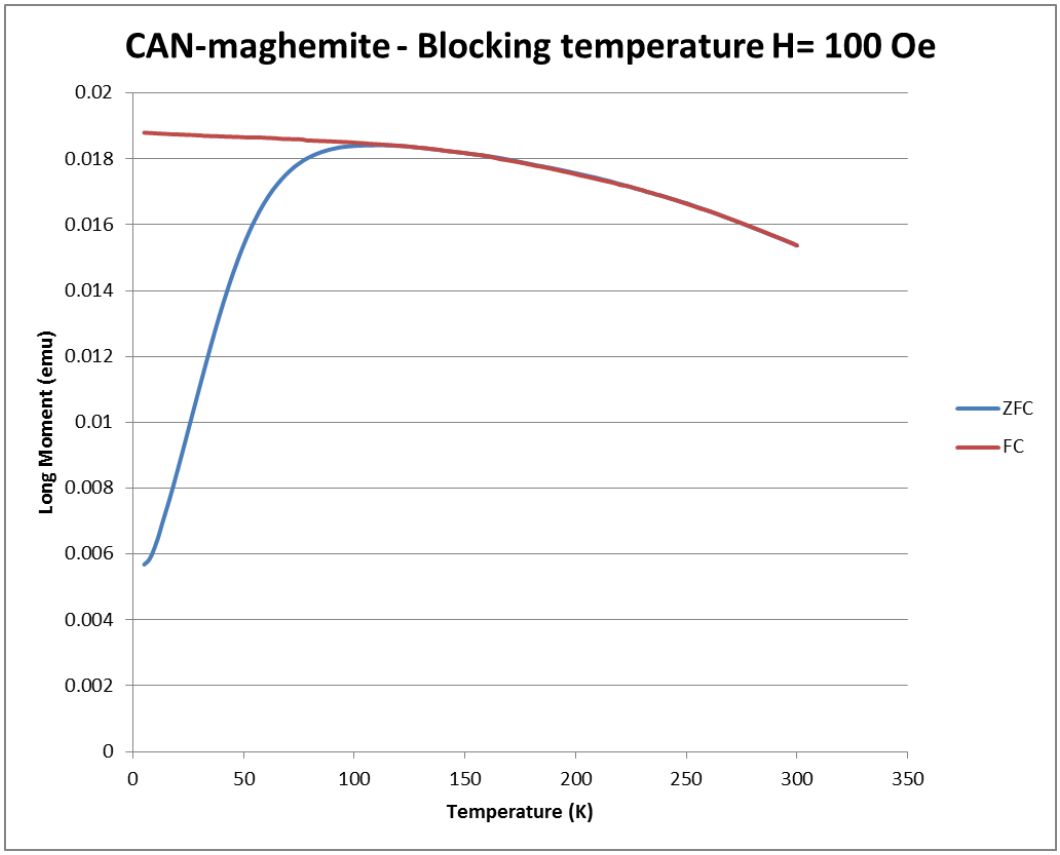


Figure SI-9. DoE-optimized ultra-small 6.61±2.04 nm-sized core $\mathbf{CAN_{DOE}}$-γ-$\mathbf{Fe_2O_3}$ **NPs (**MINITAB® 16 software profile optimizer tool output): ZFC/FC graphs (blocking temperature T_B: 109-110°K, H = 100 Oe)

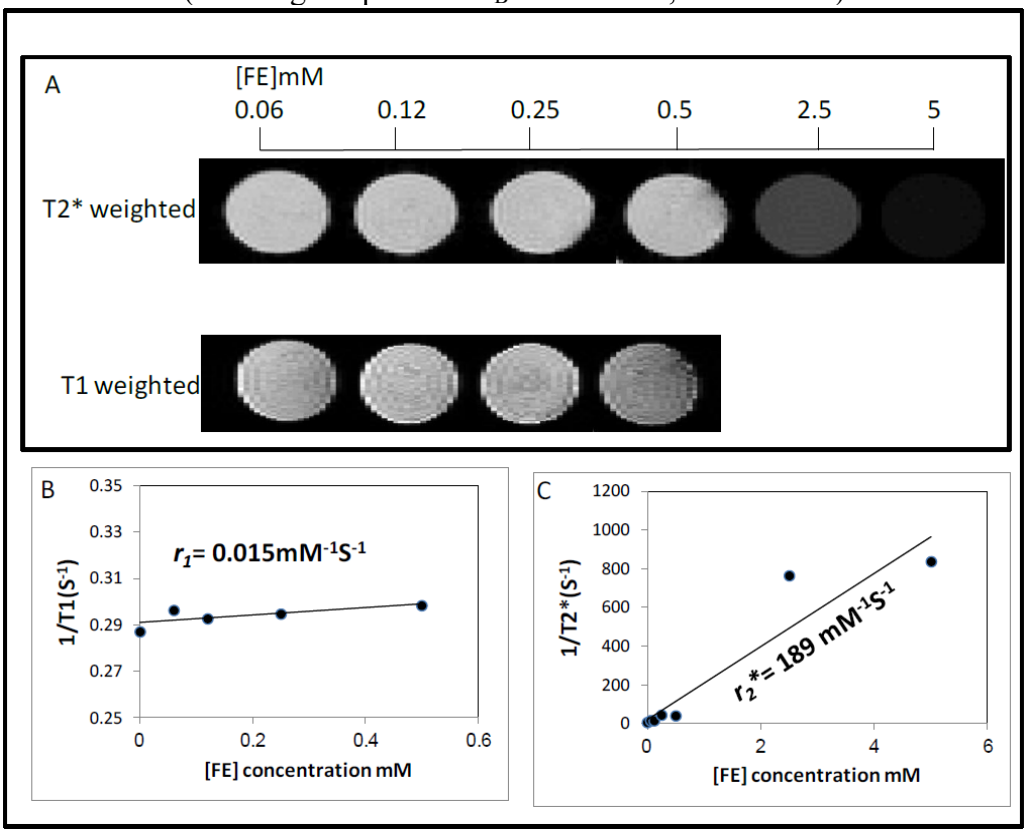


Figure SI-10. MRI relaxivities images & measurements for DoE-optimized ultra-small 6.61±2.04 nm-sized core **CAN_{DOE}-γ-Fe_2O_3 NPs (**MINITAB® 16 software profile optimizer tool output):

F. Selected characterization data of optimal *b*-PEI_{25}-CAN_{DOE}-γ-Fe_2O_3 NPs

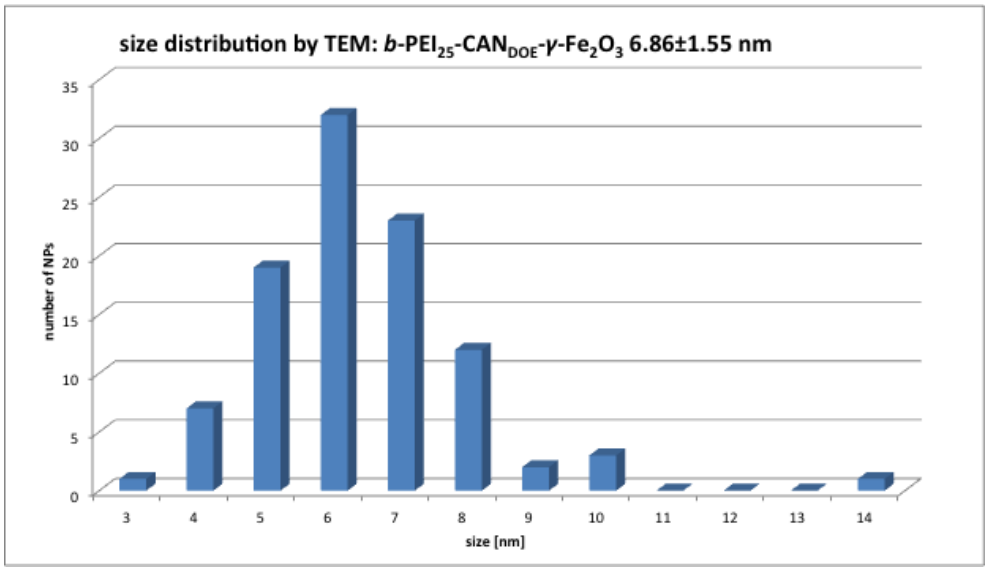


Figure SI-11. Size distribution histogram of ultra-small averaged 6.86±1.55 nm-sized **optimized *b*-PEI-CAN_{DOE}-γ-Fe_2O_3 NPs**

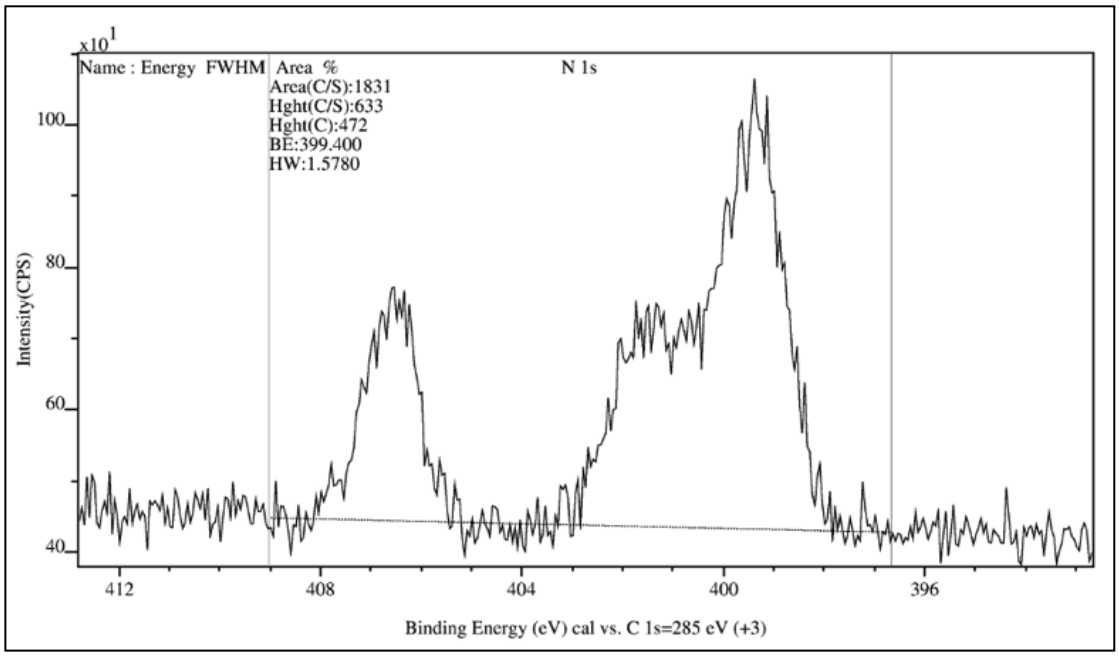


Figure SI-12. XPS analysis of optimal ***b*-PEI_{25}-CAN_{DOE}-γ-Fe_2O_3 NPs**, N 1s peaks (*nitrate anions & amine species*, BE: 406.0-407.0 & 398.0-402.0 eV respectively)

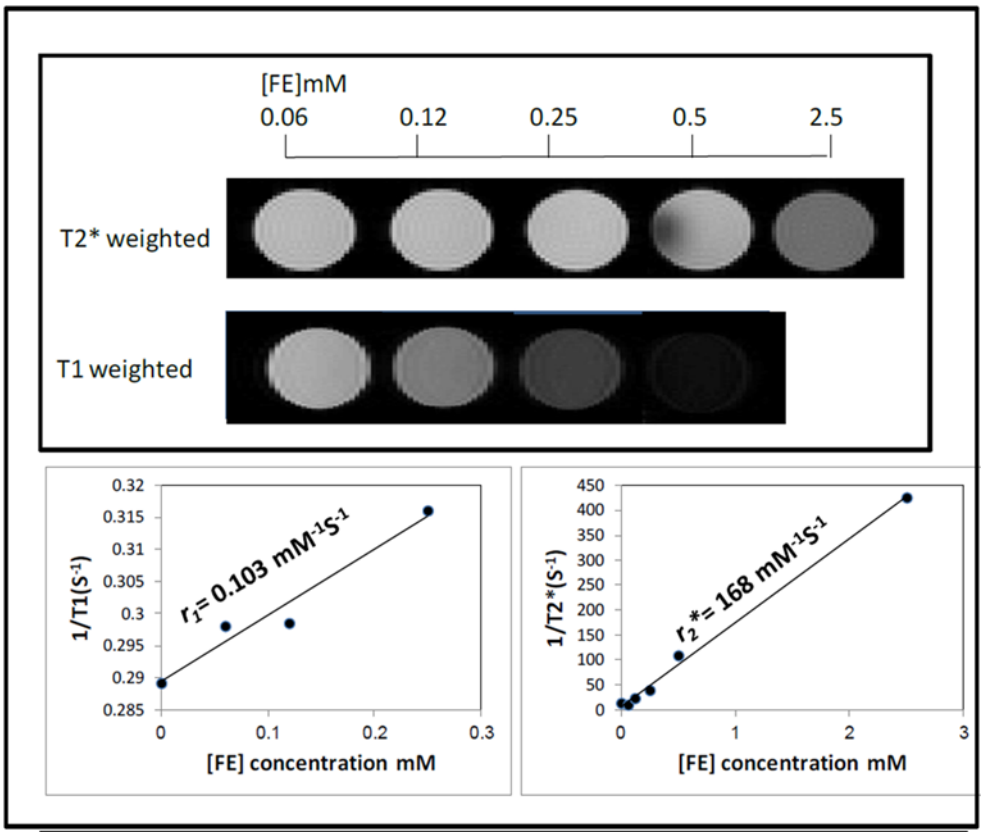
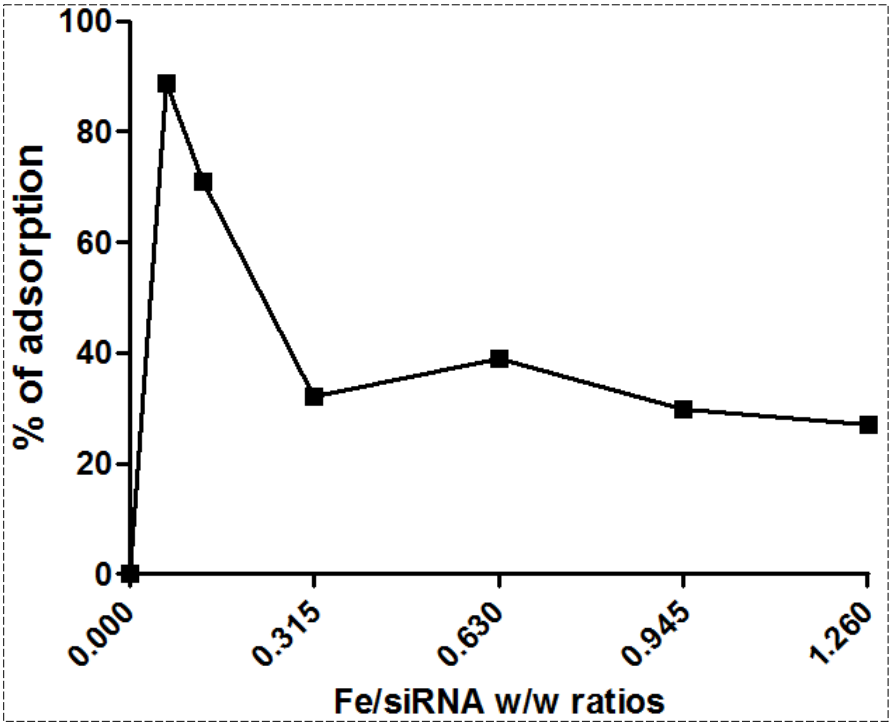


Figure SI-13. MRI relaxivity images & measurements for **optimized *b*-PEI-CAN$_{DOE}$-γ-Fe$_2$O$_3$ NPs**

G. Selected experimental procedures for nanocarrier functionalization using siRNA species for cell delivery/gene silencing

G1. Nanoparticle-mediated capture of siRNA - Illustrating example

Aqueous suspensions of former *b*-PEI_{25}-CAN_{DOE}-γ-Fe_2O_3 NPs were diluted in ddH_2O at different concentrations to reach different w/w Fe/siRNA mass ratios (0.063, 0.126, 0.315, 0.630, 0.945, and 1.260). Following appropriate calculations based on former TGA & ICP-AES data (NP batch: 0.079 mg NPs/mL suspension), these Fe/siRNA w/w ratios are equivalent to the following *b*-PEI_{25}/siRNA w/w ratios: 0.493, 0.986, 2.465, 4.930, 7.395, and 9.860. To each nanoparticle suspension and to the control tube (absence of particles), 3.0 μg of siRNA were added and incubated at room temperature for 15 min toward complex formation. After 15 min of incubation, suspensions were centrifuged at 11,000 rpm for 10 min and free siRNA was measured in supernatant with a spectrophotometer (Nanodrop 1000, Fisher Scientific, λ: 260 nm). The amount of free siRNA at each ratio was normalized to control tube (Fig. SI-15 reported below).



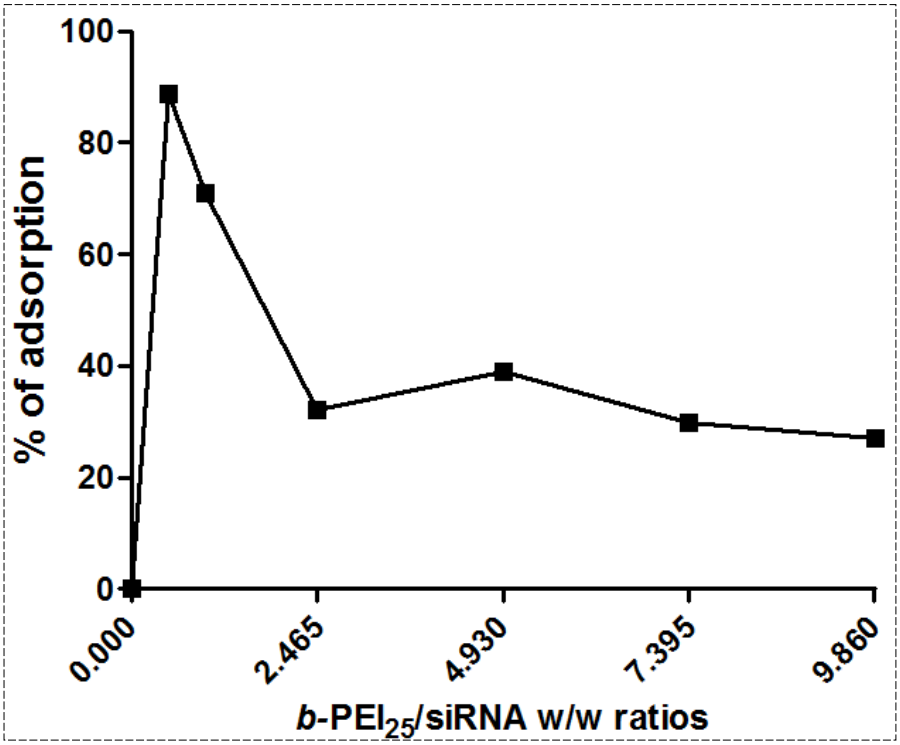


Figure SI-14. siRNA adsorption graphs by optimal ***b*-PEI$_{25}$-CAN$_{DOE}$-γ-Fe$_2$O$_3$ NPs** at various increasing (i) Fe/siRNA (*top*) & (ii) *b*-PEI$_{25}$/siRNA (*bottom*) w/w ratios

G2. Gene silencing experiments - Cell culture medium

U2OS human osteosarcoma cells have been obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in a Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 µg/mL penicillin, 100 U/mL streptomycin, 2mM *L*-glutamine and 25mM HEPES (Biological Industries Ltd., Israel). Cells were grown at 37^{o}C in 5% CO_2.

G3. Luciferase U2OS human osteosarcoma cell line

U2OS cells were transfected by electroporation with (i) 5 µg psiCHECK-2 *Firefly* and *Renilla* luciferase expression vector (Promega), and (ii) 0.25 µg pPUR puromycin resistance vector (Clontech). Plasmids were mixed with U2OS cells (5×10^6 cells in 500 μL phosphate-buffered saline), and the mixture was incubated at 4°C for 5 min and, then electro-pulsed with a Gene Pulser Xcell apparatus (Bio-Rad) at 170 V and 450 μF. After electroporation, cells were re-suspended in a complete medium and incubated at 37°C in 5% CO_2. Subsequently, cells with incorporated plasmids were selected with 1 μg/mL puromycin (Invivogen) and maintained in the same complete

medium for several weeks. Finally, clones were isolated and maintained in a complete medium with 1 μg/mL puromycin for processing during additional supplementary weeks.