Supporting Information for
Knocking (Anti)-Sense into Cells – The Microsphere Approach to Gene Silencing

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Preparation of siRNA Microspheres

200 and 500 nm amino microspheres were firstly functionalised with a modified PEG linker as follows: Microspheres were washed and suspended in DMF (3% solid content, 0.5 mL). Separately, Fmoc-amino carboxy poly(ethylene glycol) (10 equiv.) was dissolved in DMF (0.5 mL) with HOBt and DIC (10 equiv.) and mixed for 10 minutes. After this time, the solution was added to microspheres and mixed for 18 hours at 25 ºC. Subsequently, the microspheres were washed by centrifugation (13,000 rpm, 3 – 10 minutes) with DMF, methanol and water. Fmoc deprotection was subsequently achieved via treatment with 20% piperidine/DMF (3 × 20 minutes) and microspheres (**1a-b**) were sequentially washed as previously.

200 and 500 nm succinic microspheres (**2a-b**) were prepared by the addition of succinic anhydride (10 equiv.) and DIPEA (10 equiv.) in DMF and mixed at 25 ºC for 18 hours. Microspheres were then sequentially washed as described.

200 and 500 nm disulphide microspheres (**3a-b**) were prepared by the addition of carboxyethyl disulphide (10 equiv.), HOBt and DIC (10 equiv.) in DMF and mixed at 25 ºC for 18 hours. Microspheres were then sequentially washed as described.

Microspheres (**2a-b, 3a-b,** 100 µL, 3% solid content) were sterilized by U.V. radiation and with RNase Zap solution (Invitrogen) before pre-activation in MES buffer (pH 5.5) with EDAC.HCl (10 equiv.) for 2 hours at 25 ºC. The microspheres were isolated by centrifugation (as previous) and amino-siRNA (1 nmol) was then added in sterile RNase-Free water (100 µL) and mixed for 24 hours at 25 ºC.

Microspheres were subsequently isolated by centrifugation and stored in sterile RNase-Free water at 4 ºC.

siRNA Sequences: For EGFP silencing: Sense 5'-amino-C6-GCU GAC CCU GAA GUU CAU CUU-3'; Anti-sense 5'-GAU GAA CUU CAG GGU CAG CUU-3' (21).

Scrambled siRNA: Sense 5'-amino-C6-GUG AAA ACC AGG ACA AAA GUU-3'; Anti-sense 5'-CUU UUG UCC UGG UUU UCA CUU-3'.

Preparation of Fluorescein Microspheres

Microspheres (**2a-b, 3a-b**) were pre-activated in MES buffer (pH 5.5) with EDAC.HCl (10 equiv.) for 2 hours at 25 ºC before the addition of fluoresceinamine (10 equiv.) and mixing at 25 ºC for 18 hours. Microspheres were washed sequentially as previous and stored in water at 4 ºC.

Preparation of Dual-Functionalized Microspheres

PEGylated microspheres (**1a-b**), were treated with orthogonally protected Fmoc-Lysine(Dde)-OH (10 equiv.), HOBt and DIC (10 equiv.) in DMF for 18 hours at 25 ºC. Microspheres were subsequently washed as previous and Fmoc deprotected with 20% piperidine/DMF, yielding free amino residues, which were PEGylated as described above. Subsequently, Dde deprotection was afforded by treatment of microspheres with hydroxylamine.HCl (0.4 mmol) and imidazole (0.3 mmol) in NMP (1 mL) for 30 minutes – 1 hour. Microspheres were subsequently labelled with Cy5 by addition of Cy5 (5 equiv.) in the presence of HOBt (5 equiv.) and DIC (5 equiv.) in DMF and mixed for 18 hours at 25 ºC yielding **6**. After washing, microspheres were Fmoc deprotected and then coupled with carboxyethyl disulphide (yielding **7**) or succinic anhydride (yielding **8**) as described above.

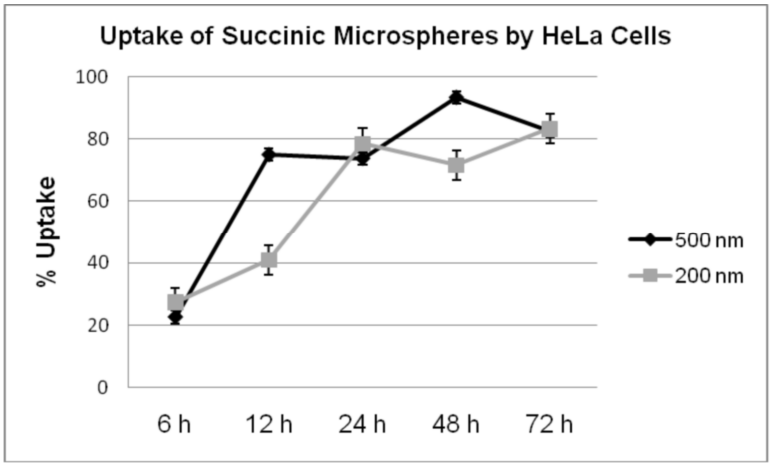
Microspheres were activated and conjugated to siRNA as described above.

Cell Cultures

HeLa and HeLa-EGFP cells were grown in Roswell Park Memorial Institute Medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin/streptomycin and 4 mM L-glutamine to 70 - 80% confluency. The cells were harvested via trypsin/EDTA and collected in growth media. They were seeded at a density of 2×10^4 cell per well in a 24-well plate and incubated at 37 ºC/5% CO_2.

HeLa Cell Uptake of Succinic-Fluorescein Microspheres

HeLa cells were cultured as described above. After 24 hours, 500 nm and 200 nm amide-fluorescein microspheres were added to the cells in fresh growth media at a concentration of 86 μg/mL. After 6, 12, 24, 48 and 72 hours the cells were detached by trypsination, collected in media and pelleted by centrifugation (1200 rpm, 4 minutes). The cell pellet was then resuspended in 0.2% trypan blue in Hank's Balanced Saline Solution (HBSS) for analysis by flow cytometry on a FACS Aria system (BD Biosciences). Excitation was made at 488 nm and emission collected with a 530/30 band pass filter. FACS Diva software was used for analysis. The % uptake is summarized in Graph S-1.



Graph S-1. Uptake of 200 and 500 nm amide-fluorescein microspheres by HeLa cells over 72 hours. % Uptake is calculated as the total % of the cell population that contains a fluorescent microsphere, where 0% uptake is untreated cells.

Disulphide Cleavage with Glutathione

200 and 500 nm disulphide-fluorescein beads (Figure S-1) were treated with 10 mM glutathione in phosphate buffered saline (PBS, pH 7.4) for 1, 6 and 24 hours. Cleavage was assessed on the reduction in fluorescence intensity of the microspheres compared to untreated control beads (Graph S-2).

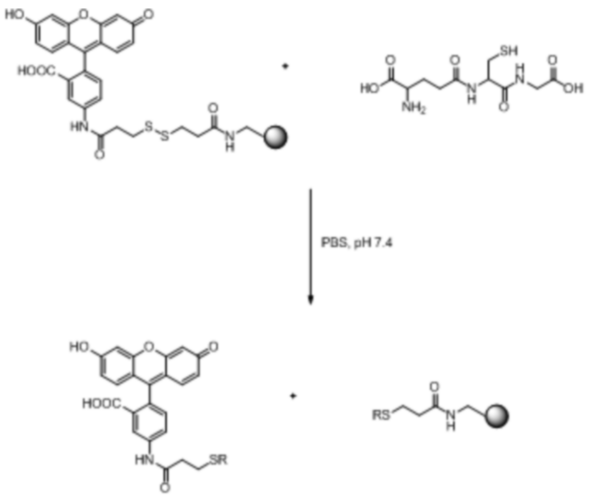
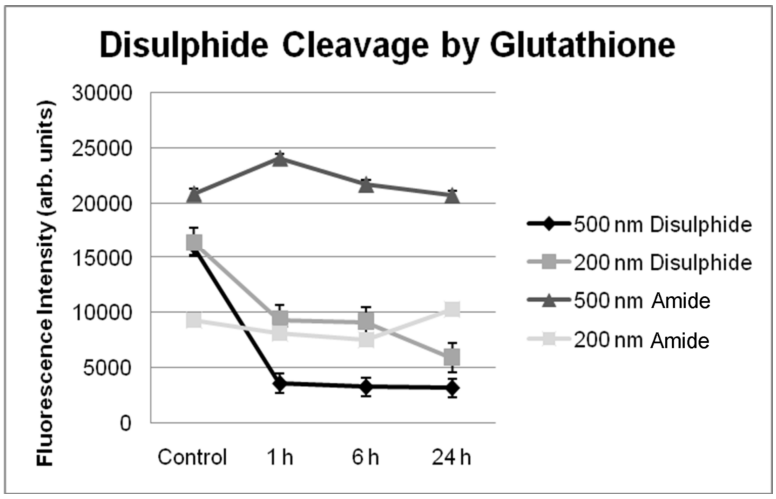


Figure S-1. Cleavage of disulphide-fluorescein beads by treatment with glutathione



Graph S-2. Cleavage of disulphide-fluorescein beads by treatment with glutathione over 24 hours.

For *in vitro* analysis, HeLa cells (not expressing EGFP) were cultured as described and disulphide-fluorescein microspheres (200 and 500 nm, 86 μg/mL) were added. Microscopy was performed after 6, 12 and 24 hours.

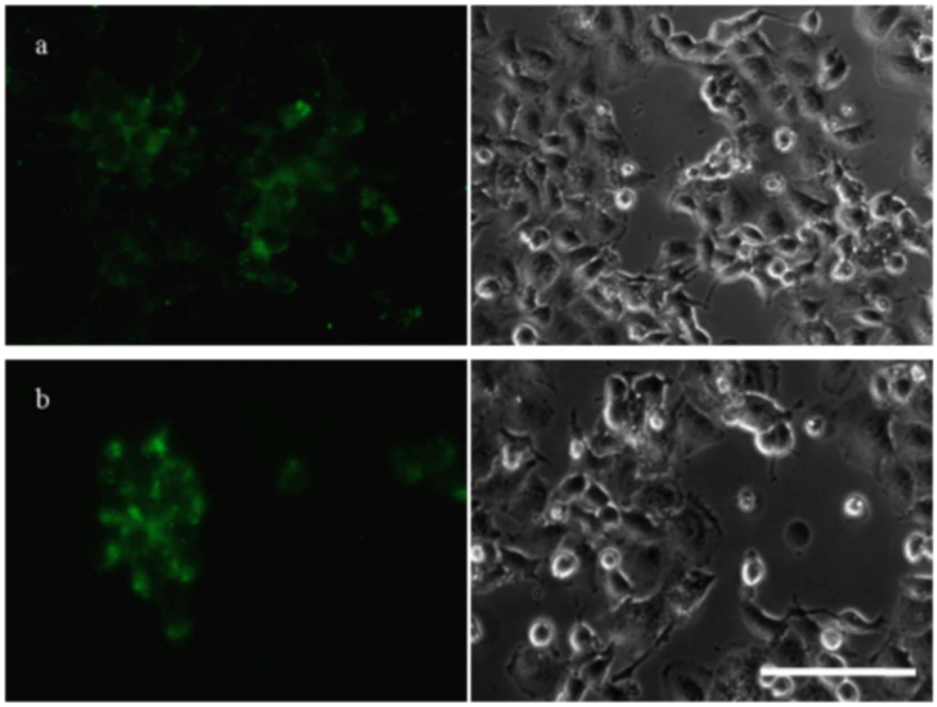
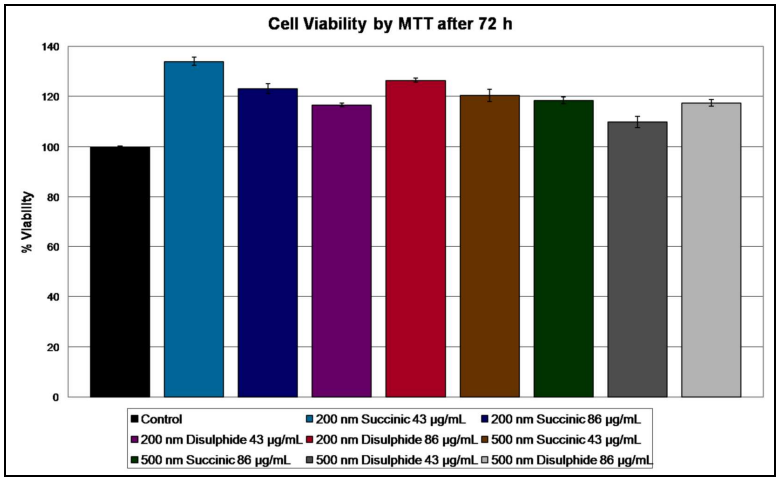


Figure S-2. Microscopy of HeLa cells incubated with **a.** 500 nm disulphide-fluorescein microspheres (**4b**); **b.** 200 nm disulphide-fluorescein microspheres (**4a**). Images taken after 12 hours (scale bar is 100 μm).

MTT Assays of HeLa-EGFP cells with siRNA Microspheres

HeLa-EGFP cells were cultured as described above. They were seeded into a 96-well plate in growth media. 200 and 500 nm amide and disulphide siRNA microspheres were added to fresh phenol-red free growth media at day 1, day 2 and day 3. After 72 hours after the first microsphere addition, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in phenol red-free media (5 mg/mL, 10 μL per well) was added and the cells incubated for a further 5 hours. After this time, MTT solubilization solution was added (10% Triton-X, 0.1 N HCl in isopropanol, 100 μL) and the well plate was gently shaken overnight. Analysis was made at 570 nm. See Graph S-3.



Graph S-3. Toxicity by MTT assay after 72 hours with siRNA microspheres in HeLa-GFP cells

Cell Staining

Staining with Hoechst 33342: Hoechst 33342 (1 µg/mL) was added in PBS (pH 7.4) to cell cultures previously washed with PBS and incubated at 25 ºC for 10 minutes. Cell cultures were washed sequentially with PBS.

Staining with Alexa Fluor® 568-Phalloidin: Cells stained with Hoechst 33342 were fixed in 3% *p*-formaldehyde solution in PBS for 20 minutes. Cultures were then washed with PBS and Alexa Fluor® 568-Phalloidin (1 unit) added in PBS (200 µL) supplemented with 1% Bovine Serum Albumin. Cells were incubated at 25 ºC for 20 minutes before washing with PBS and finally stored in PBS at 4 ºC.

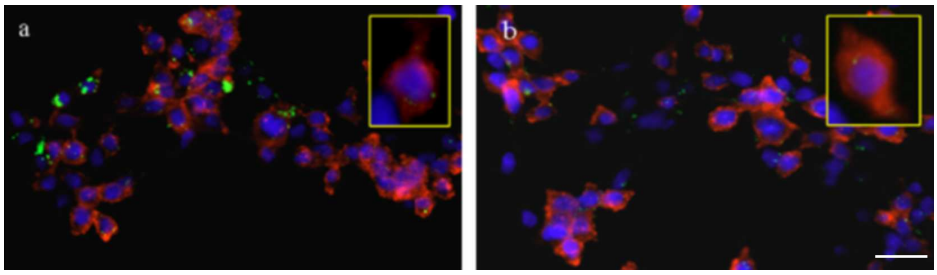
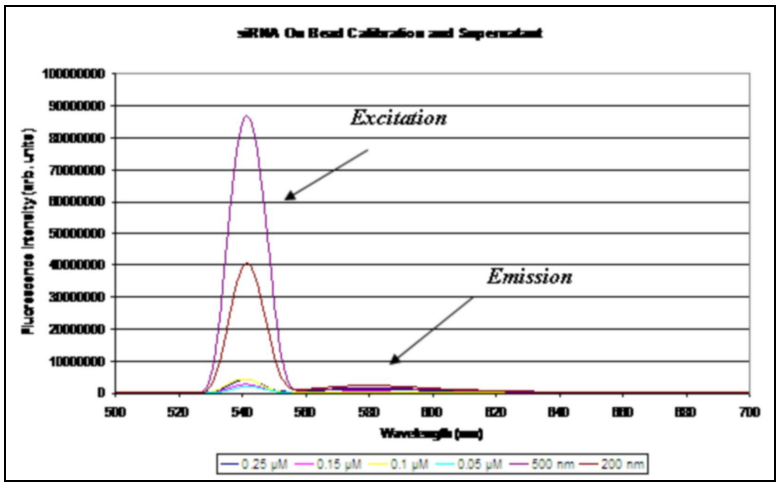


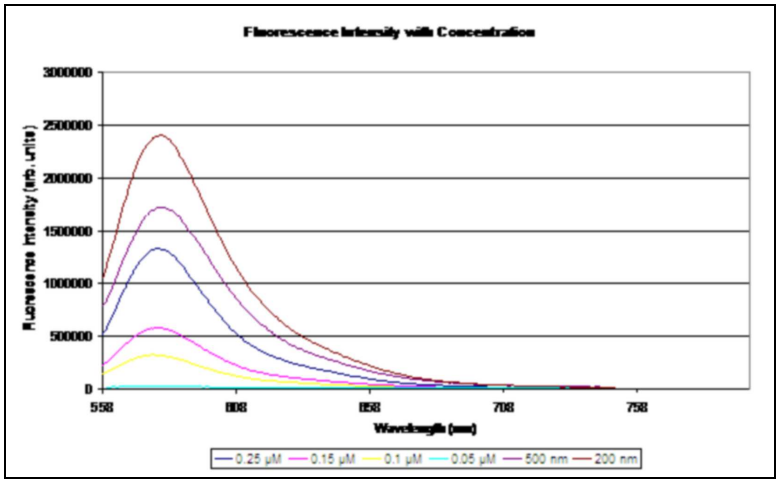
Figure S-3. Uptake of fluorescein labelled succinic **a.** 500 and **b.** 200 nm microspheres by HeLa cells. Actin filaments stained with Alexa Fluor® 568-Phalloidin and nuclei stained with Hoechst 33342. Inset is 2× magnification. Scale bar is 100 µm.

Analysis of siRNA Loading by Spectrofluorometry

The supernatant was collected from the siRNA-bead coupling by centrifugation (13,000 rpm, 2 minutes). Calibration solutions were prepared at concentrations of 0.25, 0.15, 0.1 and 0.05 µM TAMRA labelled siRNA in sterile RNase-free water and analysed on a FluoroMax spectrofluorometer and compared with the supernatant from the coupling reactions exciting at 542 nm and collecting emission from 500 – 750 nm. The emission maximum was found at 578 nm (Graph S-4 and S-5).

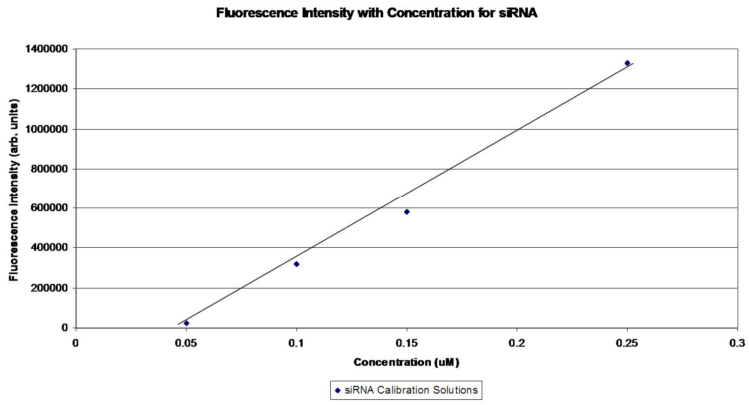


Graph S-4. Fluorescence excitation and emission collected by spectrofluorometry of calibration solutions and coupling supernatant, exciting at 542 nm and collecting emissions over 500 – 750 nm.



Graph S-5. Fluorescence emission only collected by spectrofluorometry of calibration solutions and coupling supernatant, exciting at 542 nm. Emission maximum was at 578 nm.

The calibration solutions were used to correlate TAMRA siRNA concentration to the intensity of fluorescence emission, which was found to be a linear relationship (Graph S-6).



Graph S-6. Fluorescence emission collected by spectrofluorometry of calibration solutions plotted as a function of concentration.

Using the equation of the graph (Equation S-1), the fluorescence emission recorded for the supernatant of the 200 and 500 nm bead-siRNA couplings were used to calculate the concentration of siRNA present in the samples and found to be 0.4 µM for 200 nm bead-siRNA supernatant and 0.3 µM for 500 nm bead-siRNA supernatant.

$$y = 7 \times 10^6 x - 332854 \qquad \textbf{Equation S-1}$$

Therefore: $x = (y + 332854)/7 \times 10^6$

Where x is the concentration (µM) and y is the fluorescence intensity (arb. units).

The 200 nm bead-siRNA supernatant contained 4×10^{-11} moles of siRNA and the 500 nm bead-siRNA supernatant 3×10^{-11} mol of siRNA. A total of 1×10^{-9} moles siRNA was added to the beads originally thus the 200 nm beads must have retained 9.6×10^{-10} moles siRNA (96% of siRNA coupled to microspheres) and the 500 nm beads must have retained 9.7×10^{-10} moles siRNA (97% of siRNA coupled to microspheres).

The number of beads per gram of solid can be calculated by Equation S-2.

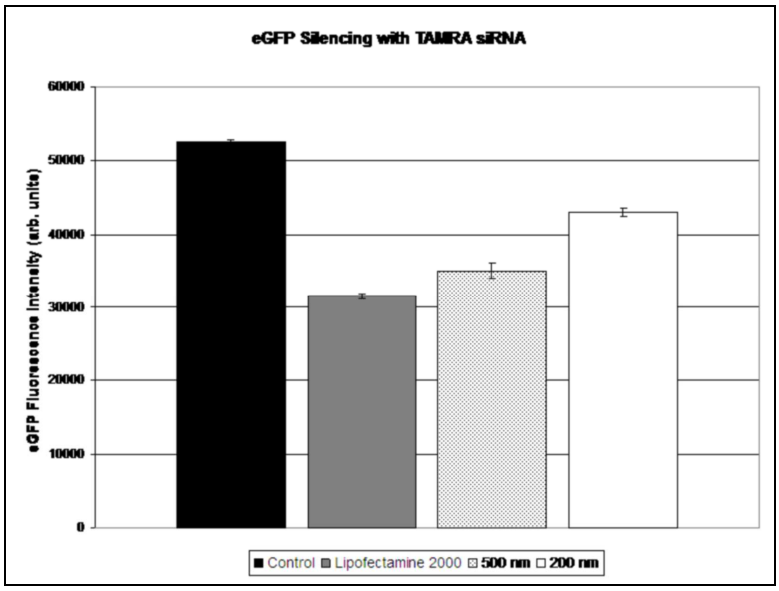
$$\textit{Number of beads/g} = 6 \times 10^{12}/(\pi \times \rho \times d^3) \qquad \textbf{Equation S-2}$$

Where ρ is the density of the microspheres (1.0 g/mL for polystyrene microspheres) and d is the diameter of the microspheres. Equation from Bangs Laboratories, Technical Note.

Thus, in the case of 200 nm microspheres, there are 2.4×10^{14} beads/g and in a 100 µL aliquot at a solid content of 3% (as used in the siRNA couplings) there is 3 mg of solid beads. This equates to 7.2×10^{11} beads, therefore each bead holds, on average, 1.3×10^{-21} moles of siRNA, which equates to 783 molecules of siRNA per bead.
In a typical culture where 86 µg/mL microspheres (or 2.1×10^{10} beads/mL) are added to cells, 1.3×10^{-21} moles of siRNA per bead equates to 9.4×10^{-12} siRNA per culture. Given the cell culture volume is 350 µL, this gives a concentration of 2.7×10^{-8} moldm^{-3}.

In the case of 500 nm microspheres, there are 1.5×10^{13} beads/g and in a 100 µL aliquot at a solid content of 3% (as used in the siRNA couplings) there is 3 mg of solid beads. This equates to 4.5×10^{10} beads, therefore each bead holds, on average, 2.2×10^{-20} moles of siRNA, which equates to 13,244 chains of siRNA per bead.
In a typical culture where 86 µg/mL (or 1.3×10^{9} beads/mL) microspheres are added to cells, 2.2×10^{-20} moles of siRNA per bead equates to 1.0×10^{-11} siRNA per culture. Given then cell culture volume is 350 µL, this gives a concentration of 2.8×10^{-8} moldm^{-3}.

Gene Silencing by Beadfection with Fluorescently Labelled and Unlabelled siRNA
HeLa-EGFP cells were cultured in the normal manner (described above) prior to the addition of siRNA conjugated 200 and 500 nm microspheres (86 µg/mL beads or 28 nM siRNA per culture) in serum free media. After 24 hours, the serum-free media was exchanged from complete media and EGFP expression was evaluated by flow cytometry after 24, 48 and 72 hours (results shown after 72 hours) in 2% FBS/PBS. A positive control was established with Lipofectamine 2000 used as according to the manufacturer's instructions using 28 nM siRNA.

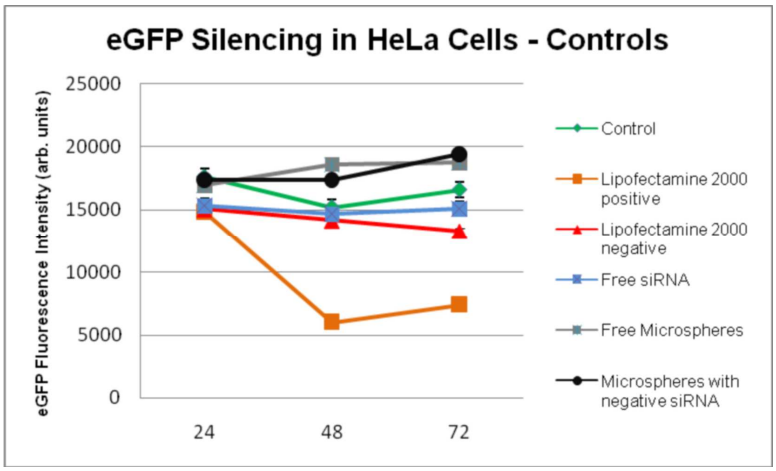


Graph S-7. EGFP silencing in HeLa-EGFP cells after 72 hours by 500 and 200 nm microspheres carrying TAMRA labelled siRNA via an amide linkage (concentration of beads: 86 µg/mL; concentration of siRNA: 28 nM).

Controls for Gene Silencing

HeLa-EGFP cells were incubated with a range of controls and analysed by flow cytometry (2% FBS/PBS) after 24, 48 and 72 hours.

The concentration of siRNA used was 28 nM to reflect that concentration used in beadfections. Lipofectamine 2000 was used as according to the manufacturer's instructions.

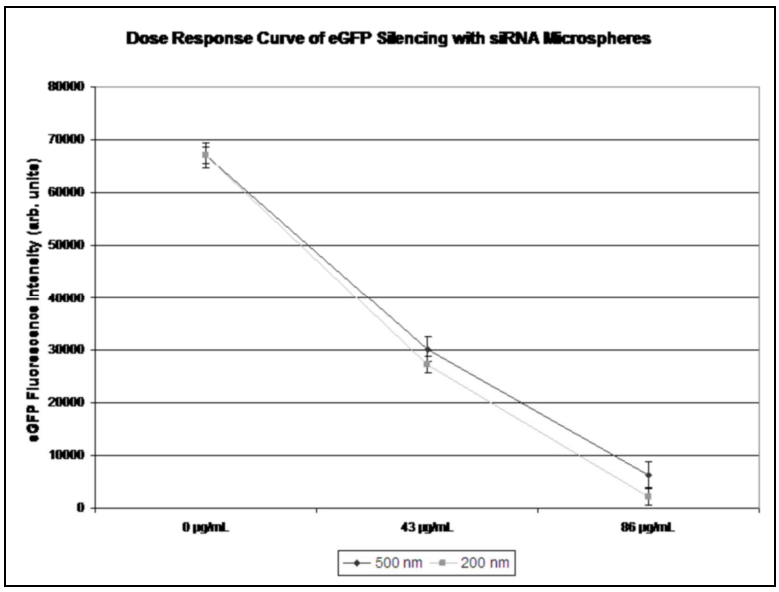


Graph S-8. Controls for gene silencing of EGFP in HeLa cells. (*Control*: untreated cells; Lipofectamine 2000 positive = siRNA transfected with Lipofectamine 2000; *Lipofectamine 2000 negative* = scrambled siRNA transfected with Lipofectamine

2000; *Free siRNA* = siRNA targeted against GFP, without a carrier device; *Free Microspheres* = 500 nm microspheres without siRNA; *Microspheres with negative siRNA* = 500 nm microspheres bound to scrambled siRNA (via an amide linkage).

Dose Response Analysis of Gene Silencing

HeLa-EGFP cells were cultured as stated above and siRNA microspheres (500 and 200 nm, amide linkages) were added to the cultures (86 µg/mL = 28 nM and 43 µg/mL = 14 nM) in serum-free media. After 24 hours, the old media was exchanged from complete media and flow cytometric analysis was carried out as described after 24, 48 and 72 hours (data not shown for 24 and 48 hours) in 2% FBS/PBS.



Graph S-9. Dose response curve following the addition of 500 and 200 nm siRNA microspheres (amide linkage, 86 and 43 µg/mL *or* 28 nM and 14 nM siRNA respectively) to HeLa-EGFP cells. Results shown are after 72 hours.