Electronic Supporting information

Magnetic pH-responsive nanogels as multifunctional delivery tool for small interfering RNA (siRNA) molecules and iron oxide nanoparticles (IONPs)

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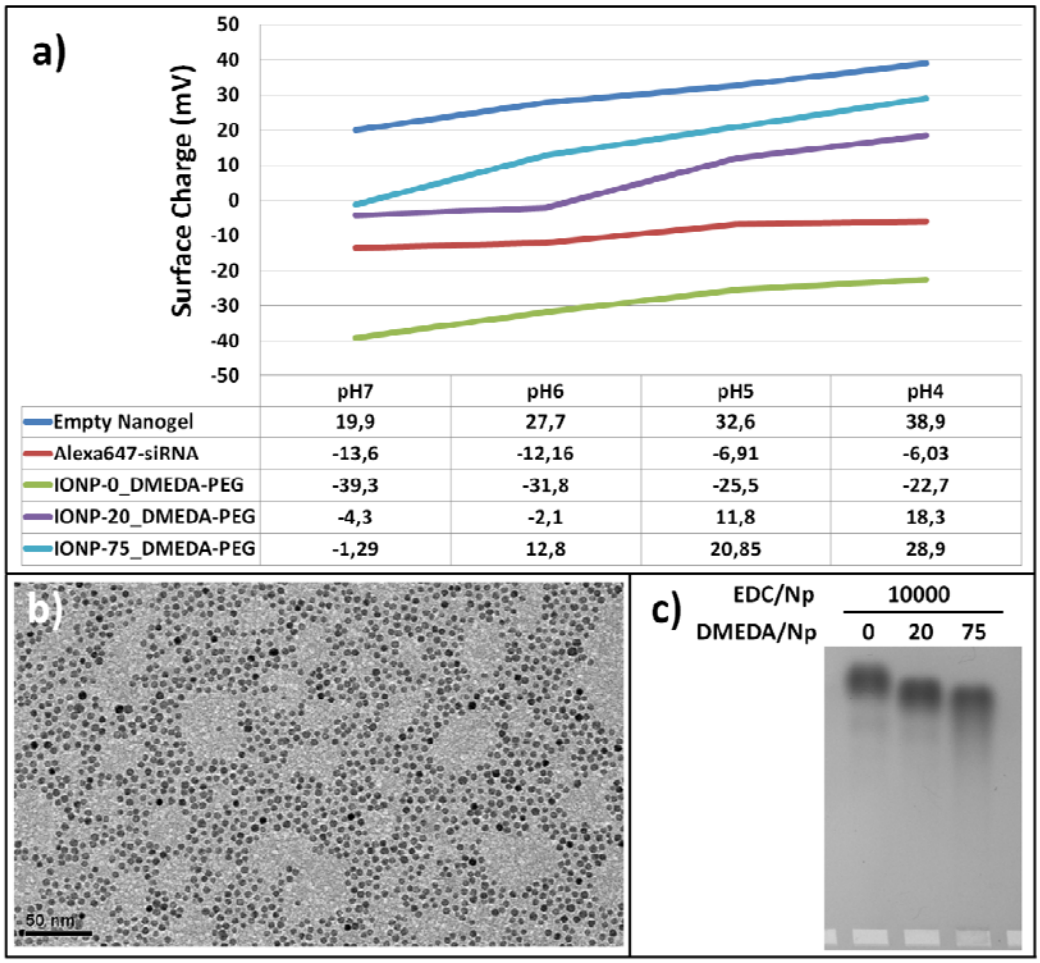
1. Reagents and Instruments

Unless specified, chemicals were purchased from Sigma-Aldrich and used without further purification. Poly (maleic anhydride–alt-*1*-octadecene), MW 20.000-25.000 was purchased from Polyscience. The HPLC purified αEGFP-siRNA oligonucleotide (target sequence 5'-GGCAAGCUGACCCUGAAGUUC-3'), modified at the 5′ end of the sense strand with Alexa Fluor 647, was purchased from Qiagen. Lipofectamine 2000 was purchased from Invitrogen. All suspensions were diluted using RNase free milli-Q water (18.2 MΩ). The pH was checked with a Crison pH-Meter Basic 20+ equipped with a microelectrode. Ultracentrifugation was performed on a Beckman Coulter Optima LE-80 K ultracentrifuge equipped with a SW41 Ti rotor. TEM images were acquired on a JEOL Jem-1011 and with a Jeol JEM 2200FS transmission electron microscopes operating respectively at an accelerating voltage of 100 and 200 kV. Zeta potential and hydrodynamic diameter were measured with Zetasizer Nano ZS90 (Malvern, USA) equipped with a 4.0 mW He-Ne laser operating at 633 nm. Spectral absorption and emission measurement were measured using respectively a Varian Cary 300 UV-vis spectrophotometer and a Cary Eclipse fluorescence spectrophotometer. True-color phase contrast cell images were acquired on Motic AE31 inverted microscope equipped with a Moticam 2500. For fluorescent Laser Scanning Confocal Microscopy (LSCM) analysis was used a Nikon Eclipse Ti microscope equipped with a A1-DUS spectral detector, 405-nm and 638-nm solid state laser lines. The concentration of the nanoparticles was determined by elemental analysis using the induction coupled plasma atomic emission spectrometer (ICP-AES iCAP 6500, Thermo). The Fe concentration was converted into nanoparticle concentration using a procedure described by us in a previously published paper[1]. The cytofluorimetric assay was performed with fluorescence activated cell sorting (FACSAriaII, BD).

2. Nanomaterials Synthesis and Nanocarrier Preparation

2.1 *Synthesis and characterization of N,N-dimethylethylendiamine/PEG functionalized IONPs*

Superparamagnetic IONPs (diameter of 6 nm) were synthesized according to the Sun method[2]. The as-synthesized nanoparticles have a capping layer of oleic acid and oleylamine and therefore they are soluble only in organic solvents. We transferred them into water by using a polymer coating procedure previously developed by us and slightly modified.[1] Briefly, the nanoparticles were mixed in chloroform with poly(maleic anhydride alt-1-octadecene) at a ratio of 150 monomer units for nm^2. Once removed the solvent by applying a reduced pressure, the polymer coated nanocrystals were dissolved in water. The carboxylic groups at the surface of the nanocrystals were then reacted with tertiary amine molecules, N,N-dimethylethylendiamine using a ratio of 20 molecules for nm^2 by EDC chemistry. In order to remove the excess of free molecules, an ultracentrifugation step was performed at 30000 rpm on a continuous sucrose gradient. Then, mono-amine-PEG molecules (750 Da) were bound to the carboxy groups at the nanoparticle surface via EDC chemistry and the un-reacted molecules were removed by performing several washing steps on Amicon (Millipore) centrifuge tubes (MWCO 30000 Da). The PEG molecules were introduced in order to make the nanoparticles more stable at different conditions of pH and ionic strength conditions. The tertiary amines were added at the nanoparticle surface for providing a zwitterionic surface to the IONPs and thus tuning their surface charge at different pH. The tuning of the surface charge at different pH allows for the loading at pH 4 and the colloidal stability at pH 7. Different ratios of amine were used and different charges at different pH were obtained. Different ratios of ternary amine/nm^2 were used thus allowing the tuning of the surface charge (Fig. SI1).



	pH7	pH6	pH5	pH4
Empty Nanogel	19,9	27,7	32,6	38,9
Alexa647-siRNA	-13,6	-12,16	-6,91	-6,03
IONP-0_DMEDA-PEG	-39,3	-31,8	-25,5	-22,7
IONP-20_DMEDA-PEG	-4,3	-2,1	11,8	18,3
IONP-75_DMEDA-PEG	-1,29	12,8	20,85	28,9

Fig. SI1. A) Surface charge characterization by Zeta Potential in water at different pH of empty pH-responsive nanogels, Alexa647-siRNA and polymer coated IONPs functionalized with different amount of N,N-dimethylethylendiamine (0, 20 and 75 molecules per nm^2 unit surface area, DMEDA) and the same amount of mono-amine-PEG MW 750 (molar ratio PEG molecules/IONP = 500, molar ratio EDC molecules/IONP = 20000). **B**) TEM images of the as prepared water soluble IONPs of about 6 nm in diameter later loaded into the pH responsive nanogels. **C**) Gel electrophoresis run (on agarose gel 1% after 1 h at 100 V) of polymer coated IONPs, and functionalized with different amount of DMEDA (0, 20 and 75 molecules per nm^2 unit surface area) and same amount of mono-amine-PEG 750.

2.2 *Synthesis of Nanogels via Emulsion Polymerization*

The polyvinyl pyridine pH-responsive nanogels were synthesized with a control over the size diameter of the nanogel below 150 nm, following a procedure published by Dupin et al.[3] and modified by our group.[4] Briefly, a mixture of 2-vinyl pyridine (2-VP, 0.25 g) and divinylbenzene (DVB, 0.013 g) was dissolved in 60 mL of water in a round-bottom flask. The pH of the resulting solution was 8.3 immediately after mixing. The flask was sealed with a rubber septum, and the aqueous solution was degassed at ambient temperature by five vacuum/nitrogen cycles. The degassed solution was constantly stirred with a magnetic stirrer and heated at 60 ± 1 °C. After 20 min, the solution of the Azobisisobutylnamide (AIBA) initiator (0.022 g in 1 mL water) was added to the flask, and after 15 min, the solution in the flask turned milky white, indicating the nucleation of the nanogels. This solution was left to polymerize for a further 2 h under stirring conditions at 60 °C, after which the flask was opened to air in order to expel the nitrogen atmosphere and to stop the reaction. In order to remove the residual monomers in solution, the 2-VP nanogel particles were dialyzed against milliQ water (60 mL against 2L, MWCO 15 kDa) with 6 times water exchange.

2.3 *Preparation and characterization of IONP/siRNA loaded pH-responsive Nanogel*

In order to load siRNA and IONPs and increase the yield of the loaded nanogels required for the cell experiments, some modifications were introduced to the previous loading reaction.[4] Briefly, to avoid the formation of aggregates, we perform the loading experiment at a much more diluted factor with respect to the previous work (0.000064 instead of 0.053 w/v (g/mL) %). Furthermore in order to protect the siRNA from the pH degradation ,the siRNA and IONP loading was performed to a slightly higher pH (pH 4.0 instead of 3.5) and the nanogels, siRNA and IONPs were kept at pH 4 for 2 hours instead of 24 as previously reported. The loading of IONPs and Alexa647-siRNA molecules (directed versus EGFP mRNA sequence) within the nanogels was performed mixing the components at the following final concentrations: 80 µg/mL of pH-responsive nanogels, 80 nM IONPs and 100 nM Alexa647-siRNA in 10 mL of pure RNase-free milli-Q water. Few drops of a HCl solution 0.1 M were added at the resulting mixture to adjust the pH to 4. Under these conditions, the permeability of the polymer changed and the nanogels were completely swollen, allowing the incorporation of the IONPs and the Alexa647-siRNA molecules. After 2 hours of gently stirring at 4°C, the pH of the solution was then slowly increased to pH 7 by drop wise addition of a 0.01 M solution of NaOH. The solution was then kept at pH 7 at 4°C for 12 hours, allowing the stabilization and the complete shrinking of the polymer. Thereafter the siRNA/IONP loaded nanogels can be easily purified from the free reagents by placing a magnet (0.2 T) to one side of the vial. The same procedure was also used in case only IONPs were loaded into the nanogels (no siRNA was present in the reaction mixture) for preparing IONP loaded nanogel. This sample was used as a negative control for the GFP interfering experiments. We used DLS and Zeta potential analyses to characterize the siRNA/IONP loaded nanogels, together with the single components. Being the charge attraction/repulsion and the colloidal dispersion the driving forces of the loading, these analyses were fundamental to control the occurred loading process. The encapsulation of the IONPs in the pH-responsive matrix, the formation of aggregates, the sample monodispersity and the cleaning from the free IONPs was monitored by TEM (fig.1a and fig. SI2). Using Alexa647-tagged siRNA, the siRNA loading efficiency was measured by photoluminescence (PL): it was calculated as the difference in fluorescence intensity between the signal of a solution at the initial Alexa647-siRNA concentration and that of the non-encapsulated Alexa647-siRNA left in the supernatant after magnetic separation of the loaded NGs. The siRNA loading efficiency was calculated as the difference of the fluorescence intensity of the initial solution and the one of the free not encapsulated supernatant, divided by the fluorescence intensity of the initial solution. The siRNA loading efficiency was of 45% (Fig. 1 C) under our experimental conditions. Confocal imaging also confirmed the Alexa647 tagged siRNA loading into the nanogels (Fig. 1 C, inset). The siRNA/IONP loaded nanogel colloidal stability was assessed in dH_2O by DLS analysis for more than 8 hours showing no change in Z average values, which were stable around 160 nm (Fig. SI3a). The same analysis, carried out also dispersing the nanogels in serum supplemented Dulbecco's Modified Eagle's Medium (DMEM), showed a ~20 nm size increase likely due to the proteins absorption on the nanomaterial surface (Fig. SI3b), as already reported.[5] In comparison with the empty nanogel, the DLS diameter versus the pH curve of the siRNA/IONP loaded nanogel showed that the loaded nanogel size started to increase already at pH 5 (instead of 4.2 of the empty nanogel), reaching a maximum swollen diameter of 500 nm, 170 nm less than the empty nanogel (fig. SI4). For the quantification of the iron amount loaded within the nanogel, the determination of Fe concentration was carried out via ICP elemental analysis.

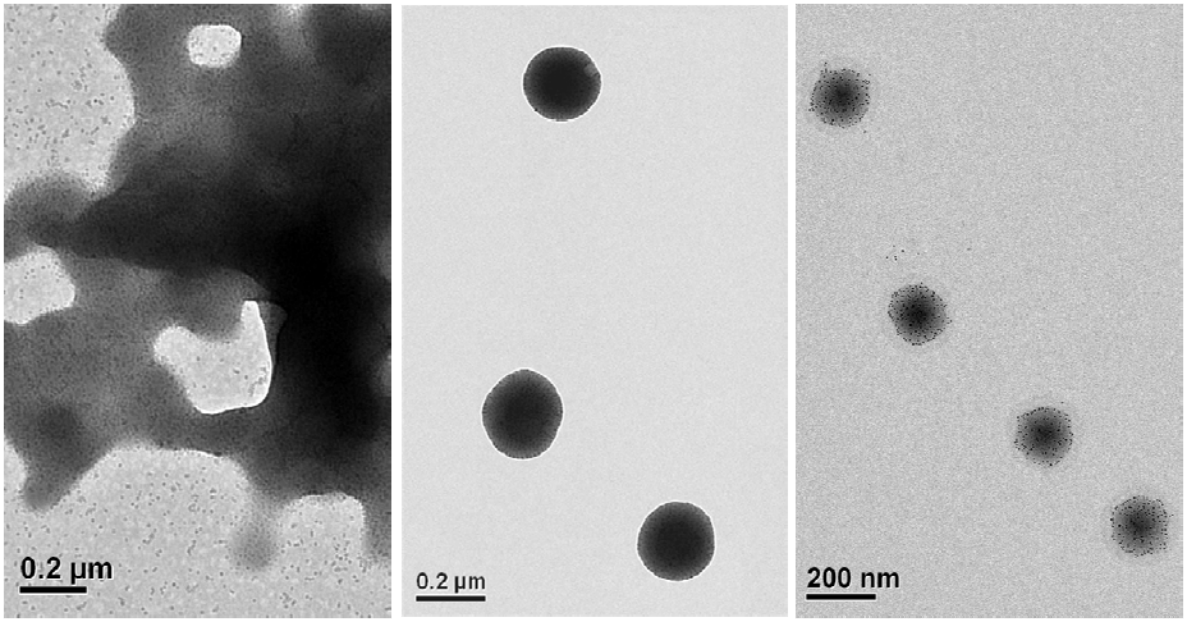


Fig. SI2. TEM images of cleaned siRNA/IONP loaded nanogels. The loading process was repeated at the same conditions changing only the functionalization of the polymer coated IONPs that was obtained using different amount of N,N-dimethylethylendiamine (left = 0, middle = 20 and right =

75 molecules per unit (nm^2) surface area) and the same amount of PEG. This sample in the middle is representative of the sample used in all the cellular experiments and is fully loaded with IONPs, shows lack of aggregations, high monodispersity and is completely cleaned from non encapsulated IONPs.

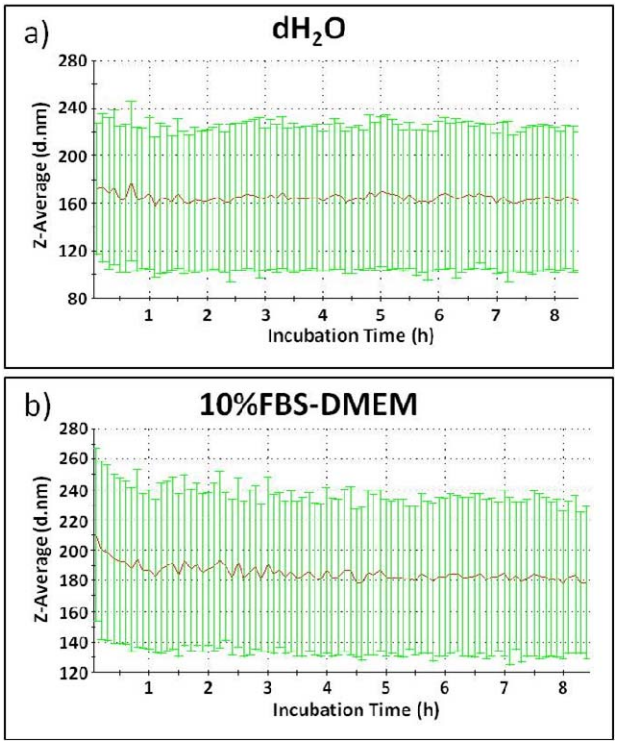


Fig. SI3. Colloidal stability test measured as DLS size of siRNA/IONP loaded nanogels as a function of the time.for the nanogels in de-ionized H_2O at 25°C (a) and in 10% FBS supplemented DMEM at 37°C (b). The measures were repeated 5 times for each time point. The DLS size in 10%FBS-DMEM medium is slightly larger than that in H_2O likely because of adsorption of serum proteins at the nanogel surface.

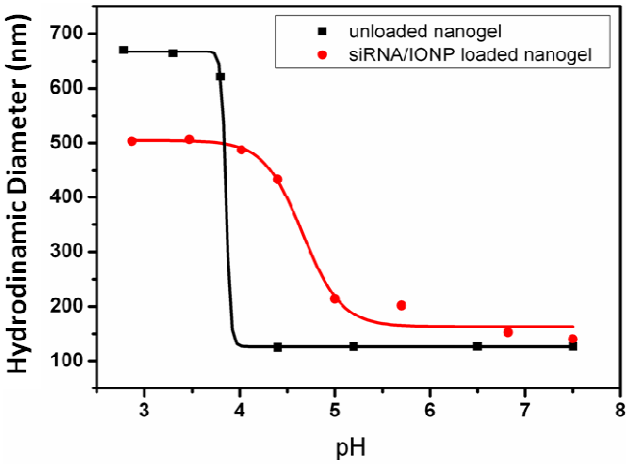


Fig. SI4. Comparison of the swelling behavior between unloaded nanogels and siRNA/IONP loaded nanogels. The hydrodynamic diameter measured by DLS are reported as a function of the pH. The pH-response of the loaded nanogel is slightly modified as the swelling start already at pH 5, while the empty nanogels swell around pH 4.2. Also at acidic pH, the loaded nanogels reach a maximum swelling diameter that is reduced with respect to that of empty nanogels (500 nm with respect to 670 nm). The swelling of the loaded nanogel at pH 4 is however still significant with respect to that at pH 7 (3 to 4 times bigger) thus allowing the payload release.

3. Cell Culture and Stable GFP Transfection

Wild type HeLa cells (HeLa WT) were grown at 37°C and under 5% CO_2 atmosphere in DMEM, supplemented with L-glutamine (2 mM), penicillin (100 units mL^{-1}), streptomycin (100 mg mL^{-1}), and 10% heat-inactivated fetal bovine serum (FBS). 24 hours before the experiments the cells were detached using a solution of 0.05% trypsin and 0.53 mM EDTA, centrifugated, resuspended in fresh complete DMEM and plated at the required density. GFP over-expressing HeLa cells (HeLa GFP) was obtained by lipofectamine transfection of pAcGFP1-N1 vector (ClonTech). After three days of transfection the cells were treated with neomycin antibiotic at increasing concentrations until 1mg/mL to select the positive GFP over-expressing cells. After several passages in culture, the percentage of GFP positive cells was increased and monitored by fluorescence-activated cell sorting (FACS) reaching a value of 90% ca.

4. siRNA/IONP loaded Nanogels Interaction with Cells

4.1 *Magnetic Enhancement of the siRNA/IONP loaded Nanogels on Cells*

The cellular uptake of Alexa647-siRNA/IONP loaded nanogels can be also magnetically driven by a magnetic field. The fluorescent signal coming from the Alexa647-tagged siRNA was useful to track the intracellular distribution and the pathway of the internalized siRNA molecules. In order to investigate the cellular uptake and the intracellular localization of the loaded pH-responsive nanogels by confocal microscopy, HeLa WT cells were seeded on a coverslip in 12 wells multiwell in 1 mL of DMEM with 10% of FBS at a density of 2 x 10^5 cells per well. After 24 hours the growing medium was replaced with a fresh complete medium containing the Alexa647-siRNA/IONP loaded nanogels at concentration of 10 μg mL^{-1} of Fe and 80 nM of siRNA with and without an oscillating magnetic field (0.2 T) placed beneath the cell plates. After 12 hours of incubation, the nanogels were removed together with the growing media. Then the cells were rinsed several times with PBS 1X, fixed with 4% paraformaldehyde in PBS 1X for 10 min at room temperature, rinsed 3 times with PBS 1x and mounted with a DAPI containing mounting medium (Vectashield). The Perls Prussian Blue stain was used for the staining of the IONPs: briefly, the cells were incubated for 30 min at room temperature with a solution of 4% potassium ferrocyanide and 4% hydrochloric acid prepared just before use. The cells were then rinsed with PBS 1X, mounted on a glass slide using glycerol and analyzed by a true-color optical microscope. Iron deposits appeared as blue precipitate thus revealing the amount and the localization of the IONP/siRNA loaded nanogels inside the cells. For the confocal microscopy analysis, the fixed and permeabilized cells were mounted on a glass slide using Vectashield mounting medium with DAPI (Vector Labs). As showed in Fig. SI6 (a,c vs. b,d), the presence of the magnetic field boosts the nanogel uptake by HeLa cancer cells, indicating that the magnetic feature can be exploited to optimize the cellular uptake.

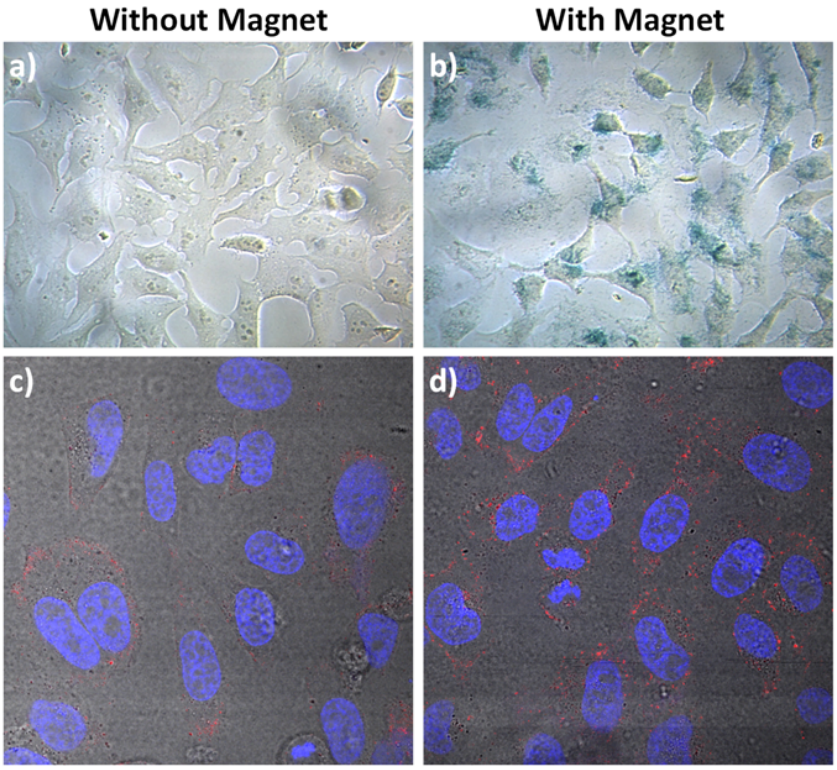


Fig. SI5. Confocal and optical microscope images show the magnetic field influence on the uptake in HeLa cells of Alexa647-siRNA/IONPs loaded nanogels. The cells were incubated in presence of the loaded nanogels for 12 h, at a concentration of 10 μg mL-1 of Fe and 80nM of siRNA, without (**a** and **c**) and with magnet (**b** and **d**) placed under the cell culture plate. The iron deposits are evidenced by Prussian blue staining (**a** - **b**). Images were acquired using an optical inverted microscope, equipped with a color camera to reveal the blue stain. Confocal fluorescent analysis (**c** - **d**) shows the intracellular amount and localization with a higher resolution and sensibility: images **c** and **d** are merged of Alexa647-siRNA (red), DAPI (blue) and of the transmitted signal.

4.2 *Flow Cytofluorimetric Analysis*

For the siRNA down-regulation assay, 3 x 10^5 HeLa GFP cells were seeded in each well of a 6 well-plate in 2 mL of complete medium. The analysis was performed on the following samples: (1) non-fluorescent wild-type HeLa, (2) untreated HeLa GFP, and HeLa GFP treated with (3) αGFP-siRNA alone (160 nM), (4) IONP loaded nanogel (20 μg mL^{-1} of Fe), (5) siRNA/IONP loaded nanogel at two concentrations (10 and 20 μg mL^{-1} of Fe, respectively 80 and 160 nM of siRNA). As positive control we used cells treated with Lipofectamine (Invitrogen) and Magnefection (Nanotherics) using in both cases 80 nM of siRNA. After the incubation time (12 hours treatment in presence of the nanocarriers + 84 hours incubation with fresh medium), the cells were rinsed with 1x PBS and detached from the growing substrate by trypsinization, then rinsed two times by centrifugation to remove trypsin and increase the cell concentration. The cell pellet was resuspended in 300 μL of 1x PBS, filtered with a 20 μm filter in order to remove the cellular aggregates and analyzed with FACS. It should be mentioned that for all the samples treated with nanogels, the higher side scatter profiles are due to the incorporation of our nanomaterial into the cells by non-specific endocytosis and by its storage into intracellular vesicles. This leads to an increase of the granularity of the cells.

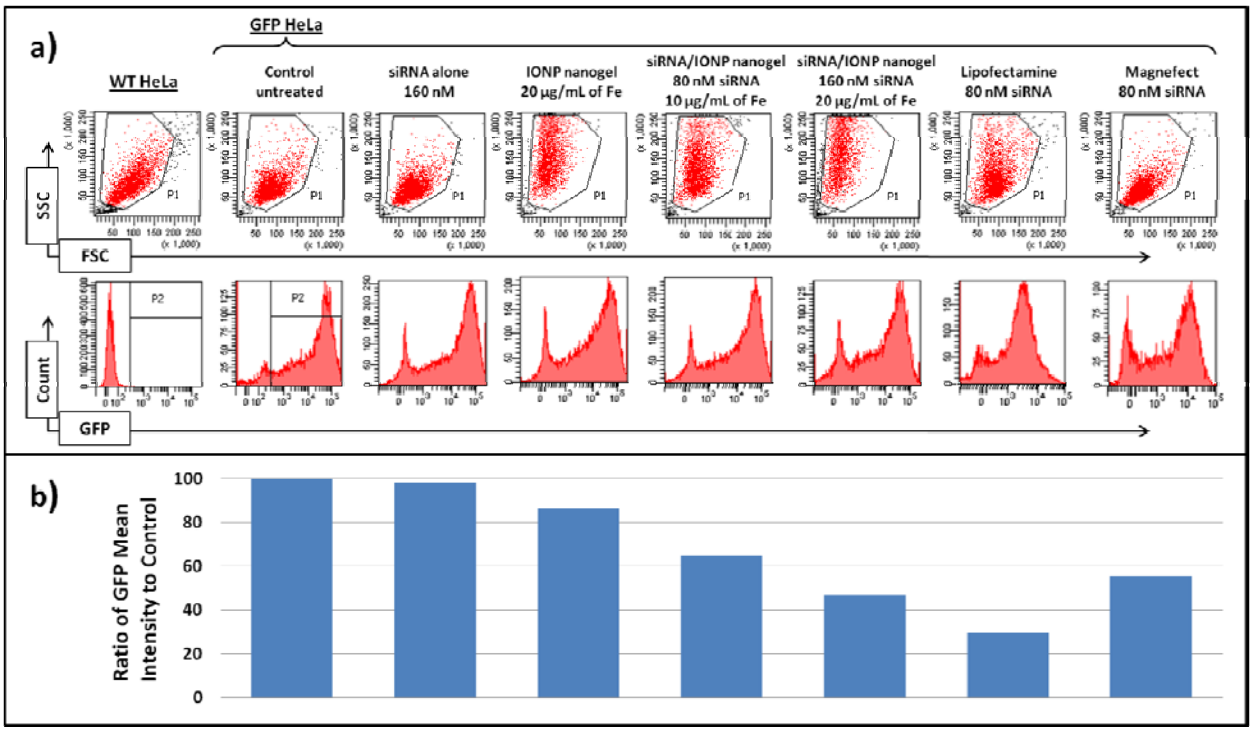


Fig. SI6. Cytofluorimetric analysis of the GFP fluorescence down-regulation in GFP over-expressing HeLa cells after the αGFP-siRNA delivery by αGFP-siRNA/IONPs loaded nanogels at a siRNA concentration of 80 and 160 nM, respect with untreated control and IONP loaded nanogel without siRNA, as negative controls, Lipefectamine (Invitrogen) and Magnefect (Nanotherics) transfected with 80 nM of siRNA were used as positive controls. In (a) for each analyzed sample, the GFP fluorescence intensity and the forward vs side scatter diagram are shown. In (b) in correspondence of each sample shown on the top part of the figure, the mean fluorescence intensity respect with the untreated control is plotted.

5.3 Cytotoxicity

To investigate the biocompatibility of our system, cytotoxicity assays were performed at 12, 24 and 96 h of treatment. The viability assay (MTT test) was performed using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide. Briefly, 5 x 10^4 cells suspended in 1 mL of medium were seeded in a 12 well multiwell and, after an incubation of 24 h at 37 °C, the medium was replaced with 1 mL of fresh serum-supplemented medium with the different transfecting agents or the siRNA alone. Cells were kept at 37°C for 12, 24 and 96 h. Then the medium was removed, the cells were washed three times with PBS, and 1 mL of fresh serum-free medium containing 1 mg mL^{-1} of MTT was added into each well. After 3 hours of incubation at 37°C the medium was removed and the precipitated dark crystals were dissolved in 1 mL of DMSO, leading to a violet solution whose absorbance at 570 nm was determined. Each assay was performed in triplicate and with the application of an oscillating magnetic field (0.2 T) under the cell plates. All the values were compared to the absorbance value of the untreated control cells. As showed in fig. SI7, while the lipofectamine have a percentage of cytotoxicity of 55-60% in both the cell lines, the siRNA/IONP loaded nanogel shows 20-30% of cytotoxicity at the highest concentration and 5-10% at the lowest concentration, in both the cell lines. It should be noticed that in cells where the GFP target mRNA was present, the siRNA loaded nanogel was actually slightly more toxic, especially after 96 h, after the siRNA escape from the endosome. This let to think that the activation of the RNAi mechanism, can affect the cell health and viability.

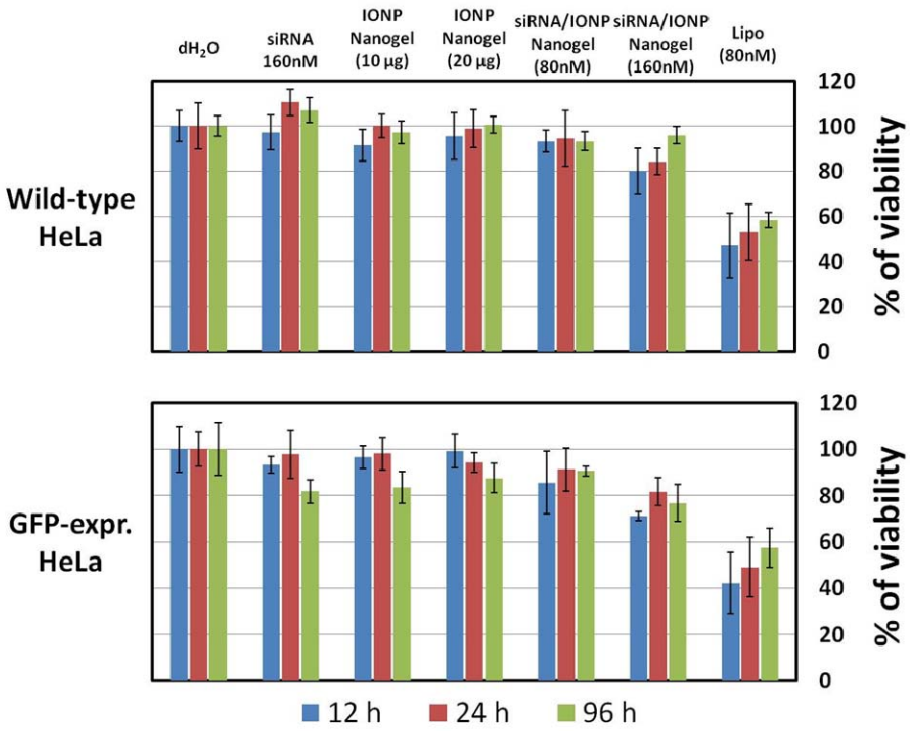


Fig. SI7. MTT Cytotoxicity Assay in wild type and GFP over-expressing HeLa cells performed after 12(blue columns), 24 (red columns) and 96 hours (green columns) of transfection with αGFP-siRNA alone (160 nM), IONP loaded nanogel (10 and 20 µg/mL of Iron), αGFP-siRNA/IONP loaded nanogel (at two different concentrations of 10 µg/mL of Iron with 80 nM of siRNA and 20 µg/mL of Iron with 160 nM of siRNA) and control with dH_2O.

5. Electron microscopy

5.1 *Cryo-sectioning*

Cryo-sectioning with respect to conventional TEM processing is well known to allow excellent membrane visibility, permitting a better structural preservation of the soft matters, like our polymer nanogels.[6] HeLa cells incubated with siRNA/IONP loaded nanogels for 12, 48 and 96 h in presence of a magnetic field (0.2 T) were fixed with 2% paraformaldehyde and 2% glutaraldeyde (PolyScience) in 0.1 M cacodylate buffer pH 7.4 for 2 hours at room temperature, washed repeatedly in PBS 1X and embedded in 12% gelatin in PBS 1X. Gelatin blocks were cut into small squared pieces and then cryo-protected by infiltration with 2.3 M sucrose overnight at 4°C. After sucrose infiltration, samples were mounted on pins and frozen in liquid nitrogen. Thin sections of about 70 nm were cut on an EM-UC6 (Leica) ultramicrotome at -120°C using a special antistatic device. Ribbons of ultrathin cryosections were collected with 1.15 M sucrose and 1% methylcellulose and placed on formvar-coated, nickel grids at 4°C. Grids were then extensively washed in water to remove sucrose and methylcellulose and then incubated 5 min in 2% uranyl acetate, briefly washed in distilled water, and then incubated for 5 min in cold 0.4% uranyl acetate and 1.8% methylcellulose to increase contrast.

5.2 *Transmission Electron Microscopy and Scanning TEM/EDXS Analysis*

Transmission Electron Microscopy (TEM) images were collected by a Jeol JEM 1011 operating at 100 KV and recorded with an 11Mp Gatan Orius SC100 Charge-Coupled Device (CCD) camera. Scanning TEM (STEM) images were acquired by a Jeol JEM 2200FS microscope, equipped with a field-emission gun (FEG) and operating at 200 kV of acceleration voltage. The Z-contrast STEM measurements were acquired using an electron probe of 0.7 nm in size, in High-Angle Annular Dark Field (HAADF) mode. To carry out spatially-resolved chemical analysis by Energy Dispersive X-ray Spectroscopy (EDXS) in STEM-HAADF mode, a Jeol JED 2300 Spectrometer equipped with a 30 mm^2 Si(Li) detector was used. The chemical quantification was performed using the Cliff-Lorimer method, which is considered as a good approximation for thin materials.

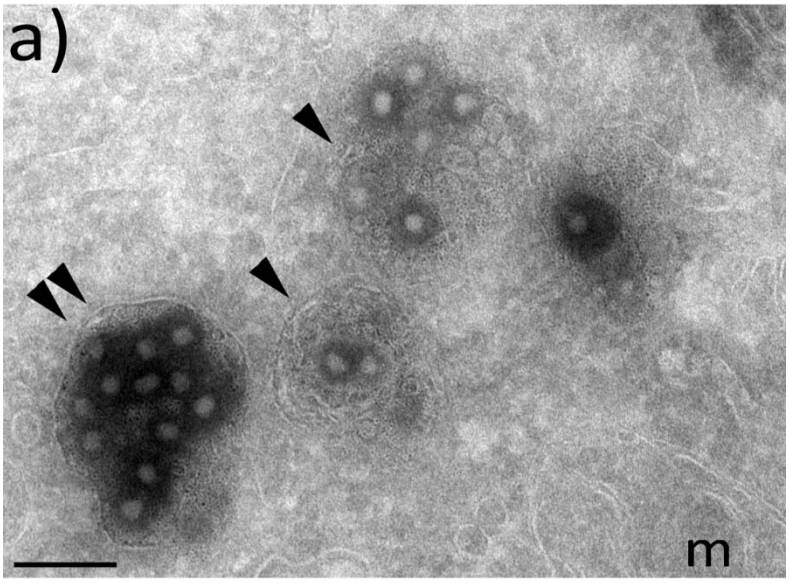


Fig. SI8. TEM image on cryosection of HeLa incubated with siRNA/IONP loaded pH-responsive NGs for 48 h. Several late endosomes (arrowheads) close to a single early endosome (double arrowheads). Abbreviation: m, mitochondrion. Scale Bar is 250 nm.

6. References

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