Supporting Information

Biocompatible iron(III) carboxylate Metal-Organic Frameworks as promising RNA nanocarriers

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1. Experimental section

1.1. Materials. Phosphate buffered saline (PBS) solution (0.01 M, pH=7.4), 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), dimethylsulfoxide (DMSO; ≥ 99.7 %), hyClone trypsin protease and heat-inactivated fetal bovine serum (FBS), from Gibco-Life Technologies. Similarly, L-glutamine (2 mM), Tris/EDTA (10 mM, pH 7.4) and SYBR® Gold stain were provided from Life Technologies. Boric acid, ammonium persulfate, electrophoresis ladder (100bp low scale ladder), Tetramethylethylenediamine (TEMED), Tris-borate-EDTA buffer (TBE), Thermo Scientific™ RNase A-DNase & protease-free (10 $mg.mL^{-1}$) and penicillin/streptomycin (100 $U.mL^{-1}$) were purchased from Fischer. Iron(III) chloride hexahydrate (97 %), 1,3,5-benzene tricaboxylic acid (trimesic acid; 95 %), DMEM medium supplemented with glutamax-1, mowiol fluorescent mounting medium (Mowiol®4-88), *p*-formaldehyde 4%, agarose, acrylamide/Bis acrylamide (30%) solution, Trizma base, glycerol, ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA, 0.5M, pH=8) and aminoterephthalic acid (98%) were purchased from Sigma-Aldrich. The reactive thiazolyl blue tetrazolium bromide (MTT) was provided by Alfa Aesar. The non-specific siRNA duplexes containing the sequences sense: 5' AGG UAG UGU AAU CGC CUU G 3' antisense: 3' CAA GGC GAU UAC ACU ACC U 5', together with the RNA oligo (miR-145) containing the sequences sense: 5'GUC CAG UUU UCC CAG GAA UCC CU 3' and the non-specific siRNA containing a fluorescent Cy5 labelled sense strand used for cellular uptake studies were purchased in Eurofins Genomics. MicroRNA Purification Kit was bought in Norgen Biotek Corporation. qScript™ microRNA cDNA Synthesis kit, PerfeCta® Universal PCR Primer and PerfeCta® SYBR® Green SuperMix, Low ROX™ were supplied by Quanta Biosciences. Primer miRNA 145 (hsa-miR-145-5p and the housekeeping small RNA control primer (RNU6) were bought at IDT and Fisher Scientific, respectively.

1.2. Synthesis of MIL-100 and MIL-101_NH_2 NPs. MIL-100 NPs were synthesized by microwave-assisted hydrothermal synthesis, according to a previously reported procedure.[1] Activation or purification of 2.5 g of MIL-100 consisted on the centrifugation (10500 rpm, 20 min) and re-dispersion of the NPs in 20 mL of distilled water and five successive times in 20 mL absolute ethanol. Further activation was carried out by re-dispersing the solid in 20 mL of a 0.1 M KF solution. The mixture was kept under magnetic stirring for 1 h 40 min under ambient conditions. Immediately after, NPs were collected by centrifugation (10500 rpm, 20 min) and washed twice with 20 mL of distilled water and once with 20 mL of absolute ethanol following the process described above. Activated MIL-100 NPs were isolated by centrifugation (10500 rpm, 20 min) and stored wet with few droplets of fresh absolute ethanol to avoid complete drying of the product. Prior to the *in vitro* experiments, NPs were exchanged in ultrapure water.

MIL-101_NH_2 NPs was obtained from a solution of 90.5 mg of aminoterephthalic acid and 135 mg of $FeCl_3 \cdot 6H_2O$ in 25 mL of distilled water placed into a Teflon-liner at 60°C for 5 min under microwave irradiation at 400W.[2,3] The obtained product was recovered by centrifugation at 10500 rpm for 10 min. With the purpose of removing the free acid, the solid was washed with absolute ethanol for 5 min, centrifuged (10500 rpm, 20 min) and kept wet. Prior to the *in vitro* experiments, NPs were exchanged in ultrapure water.

1.3. Physicochemical characterization. X ray powder diffraction (XRPD) were collected in a D8 Advance Bruker diffractometer with Cu Kα1 radiation (λ= 1.54056 angstroms) from 3 to 20º (2θ) using a step size of 0.02º and 2.5s *per* step in continuous mode. Fourier transform infrared (FTIR) spectra were collected using a Nicolet 6700 instrument (Thermo Scientific, USA) from 4000 to 400 cm^{-1}. N_2 adsorption isotherms were obtained at 77K using a BELsorp Mini (Bel, Japan). Prior to the analysis, approximately 20 mg of sample were evacuated at 37°C under primary vacuum for 3h. Thermogravimetric analyses (TGA) of the room temperature (RT) samples (5-10 mg) were analyzed on a Perkin Elmer Diamond TGA/DTA STA 6000 under O_2 atmosphere (20 $mL \cdot min^{-1}$), at heating speed of 3°C·min^{-1} for the temperature range between RT and 600°C. Particle size was monitored by Dynamic Light Scattering (DLS) on a Zetasizer Nano (Malvern Instruments; Note that is very reproducible between batches: a potential wider particle size distribution of this type of particles (with frequently 10-30% standard deviation) is probably associated to their formation mechanism (including nucleation and growth). Samples were prepared by dispersing NPs at 1 $mg \cdot mL^{-1}$ at 37ºC in the desired media by the use of an ultrasound tip (30% amplitude for 2 min; Digital Sonifer 450, Branson). NP size evolution was also monitored before and after the incubation of NPs with the siRNA. The. Fourier transform infrared (FTIR) spectra were collected in a Nicolet 6700 instrument from Thermo Scientific.

1.4. siRNA encapsulation into MIL-100 and MIL-101_NH_2 NPs. For the entrapment of small interfering RNA (siRNA) into the MIL-100 and MIL-101_NH_2 NPs were previously suspended in aqueous solutions (1 $mg \cdot mL^{-1}$, note here that NPs are weighted wet based on the wet:dry ratio previously determined from NPs dry at 100ºC overnight),[4] adjusting the pH to either 2 or 4 using a HCl 0.1 M solution. Then, 125 µL of a siRNA aqueous solution (at a concentration of 0.1 $mg \cdot mL^{-1}$) were added to 125 µL of the previously prepared NPs suspension (keeping a molar ratio 20:1). The resulting suspension was magnetically stirred at room temperature (RT) for 1 h. The siRNA loaded nanoMOFs were recovered by centrifugation at 14500 rpm for 15 min and kept wet.

1.5. Binding Assay.

The association efficacy was also indirectly determined by quantifying the amount of non-associated siRNA in the supernatant, collected by centrifugation (14000 rpm, 15 min) upon the siRNA association. The amount of free siRNA was determined by fluorescence spectroscopy, quantifying the SYBR®Gold-labelled siRNA (Table S1). The fluorescence spectra of siRNA-loaded NPs were performed on RNase free H_2O medium to determine the excitation (λ_{ex}) and emission (λ_{em}) wavelengths, which were respectively λ300 and 537 nm, being collected on an Envision Multidetector (Fluorescein High Precision Monocromator, Perkin Elmer). The calibration curves of siRNA in RNAse free H_2O were obtained in the range of concentrations from 0.25 to 2 $\mu g \cdot mL^{-1}$ with regression factors > 0.99.

Note that the differences from theoretical-experimental RNA loading could be because no steric limitations were taken into account in the theoretical calculations in order to simplify the process.

1.6. RNA Enzyme Degradation Stability.

The siRNA entrapment into the MIL-100 and MIL-101_NH_2, NPs was performed as explained in the section 1.4. The recovered siRNA loaded nanoMOFs by centrifugation was resuspended with 92.5 µL of RNAse-free water and 10 µL of the enzyme solution (endoribonuclease RNAse A: Thermo Scientific™ RNase A-DNase & protease-free (10 $mg.mL^{-1}$)). The mixtures were incubated for 20 min at 37ºC and after this time, half of each suspension was centrifuged, keeping each supernatant. Negative controls of MOF only with and without enzyme were also prepared. For this purpose, 12.5 $\mu g \cdot mL^{-1}$ of non-specific siRNA of each formulation were added in a total volume of 30 µL, placed in a 1% (w/v) agarose gel at 90 V for 30 min in TBE buffer (Figure S4). For visualization, 2 µL of SYBR®Gold (in 1:1000-fold dilution of stock dye solution), a fluorescent cyanine dye used for staining the RNA, were added in each well. The nucleic acid gels were detected by the fluorescence of SYBR®Gold using Gel Doc™ XR+ system.[5]

1.7. Colloidal stability test.

NanoMOFs and RNA@nanoMOFs were dispersed at 1 $mg \cdot mL^{-1}$ by using an ultrasound tip in different media (water, PBS-FBS (10%) and DMEM). Colloidal stability was evaluated by dynamic light scattering (DLS; Zetasizer Nano, Malvern Instruments) following the evolution of the particle size and the ζ -potential at pH 2 and pH 4 maintaining the Tª at 37ºC before and after the siRNA association.

1.8. *In vitro* cell studies.

1.8.1. *Cells and culture.* SW480 cell line (ATCC®CCL-228™) was maintained in DMEM (Dulbecco´s Modified Eagle) medium supplemented with glutamax-1 with 10% of heated-inactivated FBS and 1% penicillin/streptomycin at 37°C in a humidified 5% CO_2 atmosphere and passaged twice a week (at 80% of confluence) at a density of 5 x 10^4 cells *per* cm^2.

1.8.2. Cytotoxicity studies. The cytotoxic activity of MIL-100 and MIL-101_NH_2 NPs was analyzed by the MTT assay.[6,7] Adherent SW480 cells were seeded 24h prior to the assay in 96-well plates at a density of 1x10^4 cells *per* well in DMEM supplemented medium. The treatments were prepared at a 3-fold higher concentration (due to a direct 1/3 direct dilution in the well, as 50 µL of the NP solutions were added to a final volume of 200 µL *per* well). MIL-100 and MIL-101_NH_2 NPs solutions were incubated with the cells at different concentrations (from 50 to 1200 $\mu g \cdot mL^{-1}$) and kept at 37°C with a 5% CO_2 atmosphere. The cytotoxicity was determined upon 24h incubation of the systems, by adding the MTT reactant (0.5 $mg \cdot mL^{-1}$ in PBS, incubation at 37°C during 4h) followed by the addition of 200 µL of DMSO to each well. Absorbance was determined at λ= 539 nm under stirring.

1.8.3. Hemolysis assay. Fresh human erythrocytes were washed with PBS, followed up with a centrifugation (1000 rpm, 10 min), and several rinse cycles of the cell pellet (± 3-4 cycles). Once a clear supernatant was obtained, a suspension of 3% of erythrocytes was incubated 1:1 with different concentrations of MIL-100 and MIL-101_NH_2 NPs, from 0.5 to 0.01 $mg \cdot mL^{-1}$ at 37°C. After different incubation times (15 min and 1h), the 96-well plate was centrifuged, determining hemoglobin release in the supernatant by the absorbance measured at 537 nm. In this experiment, PBS and Triton X-100 solutions were used as negative and positive controls, respectively.[8,9]

1.8.4. Cell internalization studies. SW480 cells (ATCC®CCL-228™) were cultured in DMEM supplemented with glutamax-1, 10% of heated-inactivated FBS and 1% penicillin/streptomycin. Cells were seeded at a density of 7x10^4 cells *per* well on glass coverslips placed in 24-well plates. After 24h, the medium was replaced with 160 µL of fresh

culture medium and 160 µL of Cy5-labelled siRNA@MIL-100 / Cy5-labelled siRNA@MIL-101_NH_2 NPs, prepared at a mass ratio of 20:1 at pH=2 and pH=4. In each well, 10 µg of NPs with 1 µg of siRNA were incubated during 4h. Untreated cells and cells treated with free siRNA were included as controls.[10,11]

After the incubation time, cells were extensively washed with PBS to remove the excess of non-internalized NPs, fixed with 4% *p*-formaldehyde for 10 min and cell nuclei counterstained with the nuclear dye DAPI (1:100 in PBS, 5 min). Finally, coverslips were mounted with Mowiol fluorescent mounting medium onto glass slides and cells were examined using a Leica AOBS-SP5 spectral confocal microscope with resonant scanner, mounted on DMI 6000B inverted microscope, equipped with an Ar laser excitation lines 5 (456, 476, 488, 496, 514 nm), laser diode (561 nm), laser diode (594 nm) and blue laser diode (405 nm).

The images were collected at 405/397 (Ex/Em), 558/568 and 488/409 nm to observe the nucleus, the associated siRNA-Cy5, and Fe self-fluorescence of the NPs, respectively (Figures 1 and 2). Then, images were analyzed using the Leica Application Suite 1.7.0 built 1240, LAS AF software.[6]

1.8.5. Transfection and gene up-regulation assays. SW480 cells were seeded at a density of $250x10^3$ cells *per* well in 6-well plates. After 24 h, medium was replaced with 640 µL of fresh medium and 320 µL of loaded nanoparticles for their transfection (2 µg of non-specific siRNA and 2 µg of miR-145, equivalent to a MIL-100 and MIL-101_NH_2 NPs concentration of 20 $\mu g \cdot mL^{-1}$ / well in 960 µL of supplemented culture medium). Lipofectamine®2000 was used as a positive control for transfection in the conditions indicated by the manufacturer's protocol (Life Technologies, Paisley, UK). Once the NPs were in contact with SW480 cells for 4 h, they were replaced by fresh medium. After 72h, total microRNA was extracted using the microRNA Purification kit (Norgen Biotek Corporation) following manufacturer's instructions. Purified microRNA was eluted in a final volume of 50 µL and its concentration was measured by spectrophotometry (NanoDrop Spectrophotometer ND-1000, Thermo Scientific). Complementary DNA (cDNA) was synthesized from 1 µg of total microRNA by using the qScript™ microRNA cDNA Synthesis Kit (Quanta Biosciences). Quantitative real-time PCR was carried out using PerfeCta® MicroRNA Assays (Quanta Biosciences). The reaction mix included the cDNA samples previously obtained, SYBR green dye, universal primer and water. The mix was then amplified with the target primer miRNA 145 (hsa-miR-145-5p, IDT) and the housekeeping small RNA control RNU6 (Fisher Scientific). The PCR reaction consisted in an activation of 2 min at 95ºC followed by denaturation during 5s at 95ºC and annealing for 30s at 60ºC. The amount of PCR products was determined respect to SYBR fluorescence collected during the annealing step of each cycle (Strategene Mx3005P, Agilent Technologies). Quantitative data was analyzed by using the MxPro software, and relative quantification of miRNA 145 was derived by the delta Ct method, as described by Schmittgen and Livak.[12] Relative expression levels of miRNA 145 in each treatment group were obtained after normalizing the CT values of miRNA 145 against that of an endogenous reference gene (RNU6).

Figures and Tables

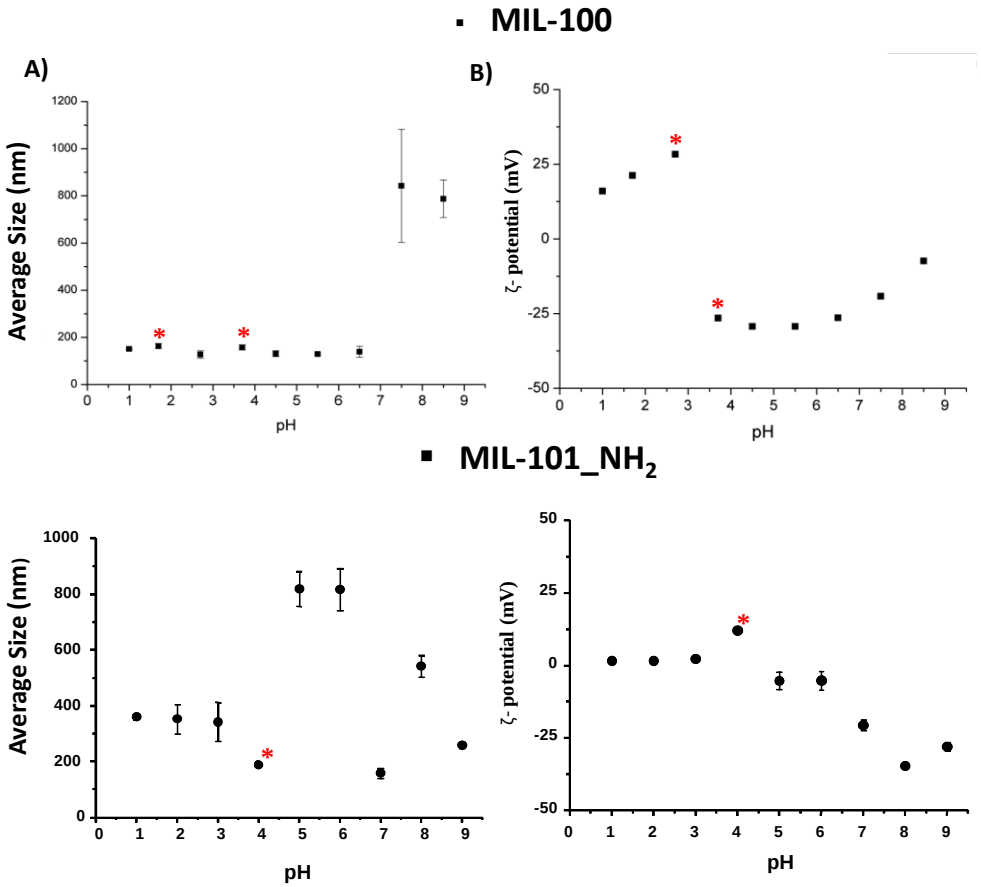

Figure S1. A) Average size (nm) and B) ζ-potential evolution (mV) of MIL-100 and MIL-101_NH_2 NPs *vs.* as a function of the pH (* highlighting the most suitable conditions of each nanoMOF).

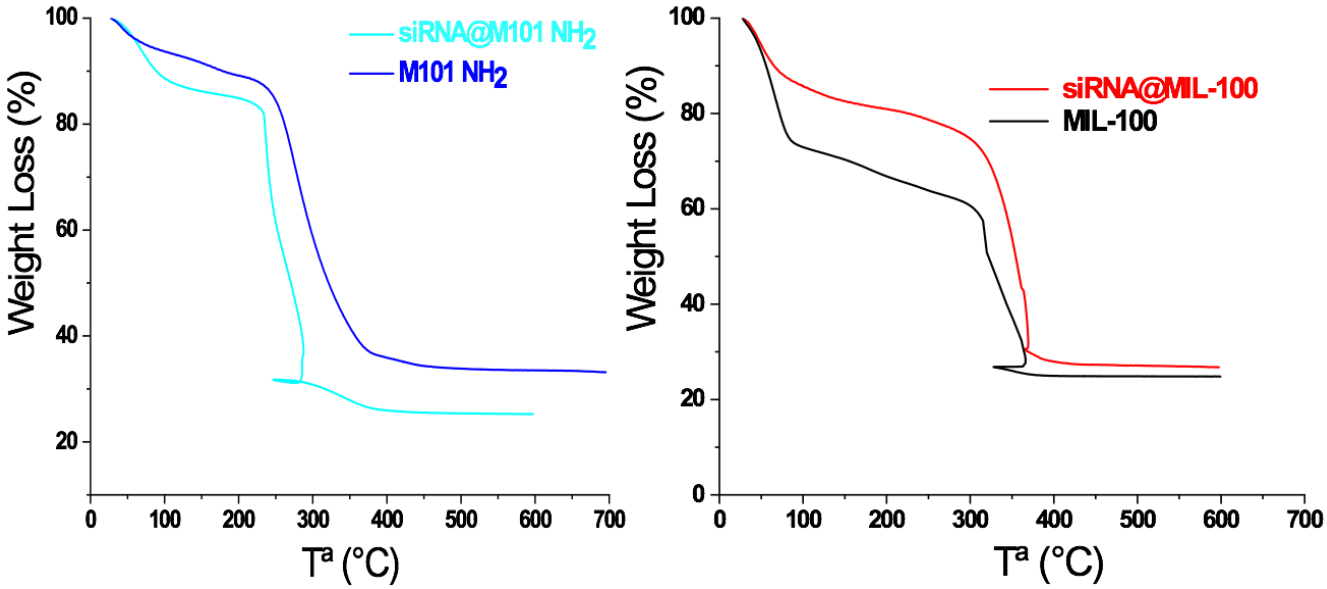

Figure S2. TGA of MIL-100 (black) and MIL-101_NH_2 NPs (blue), before and after siRNA association (red and light blue line, respectively) at pH 4.

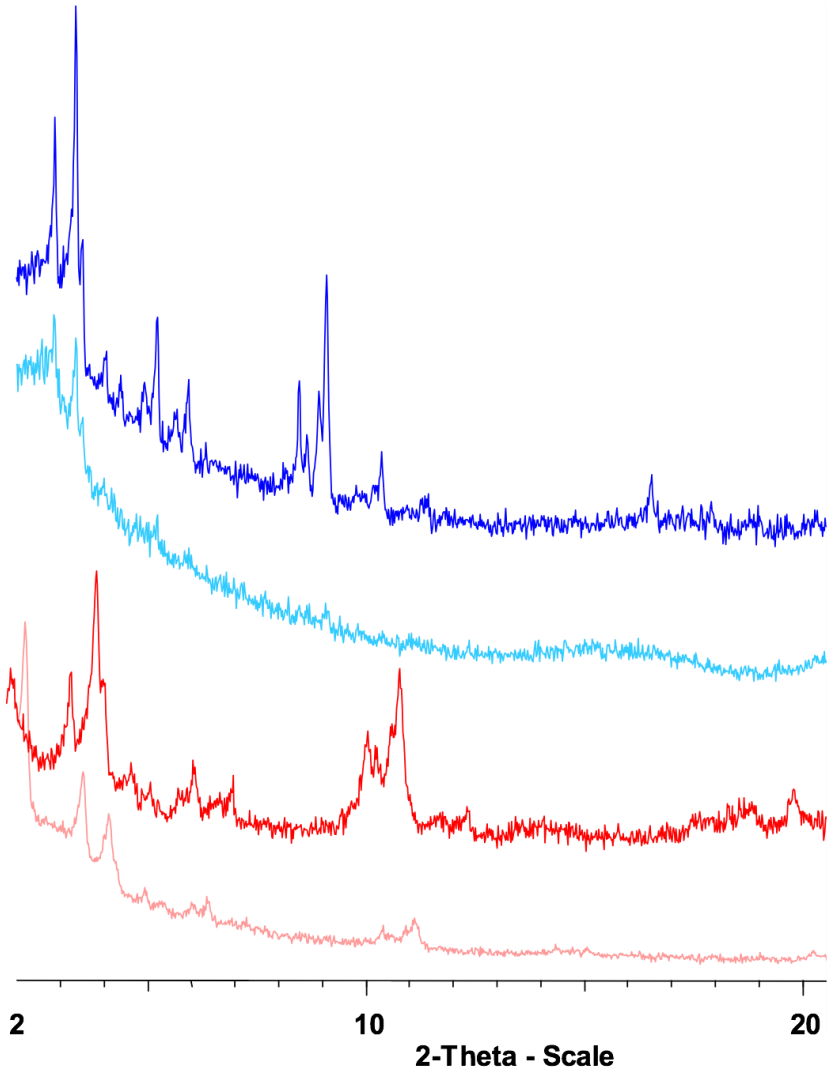

Figure S3. XRPD patterns of MIL-100 (red) and MIL-101_NH_2 (blue) NPs before (dark line) and after (light line) association of siRNA at pH 4.

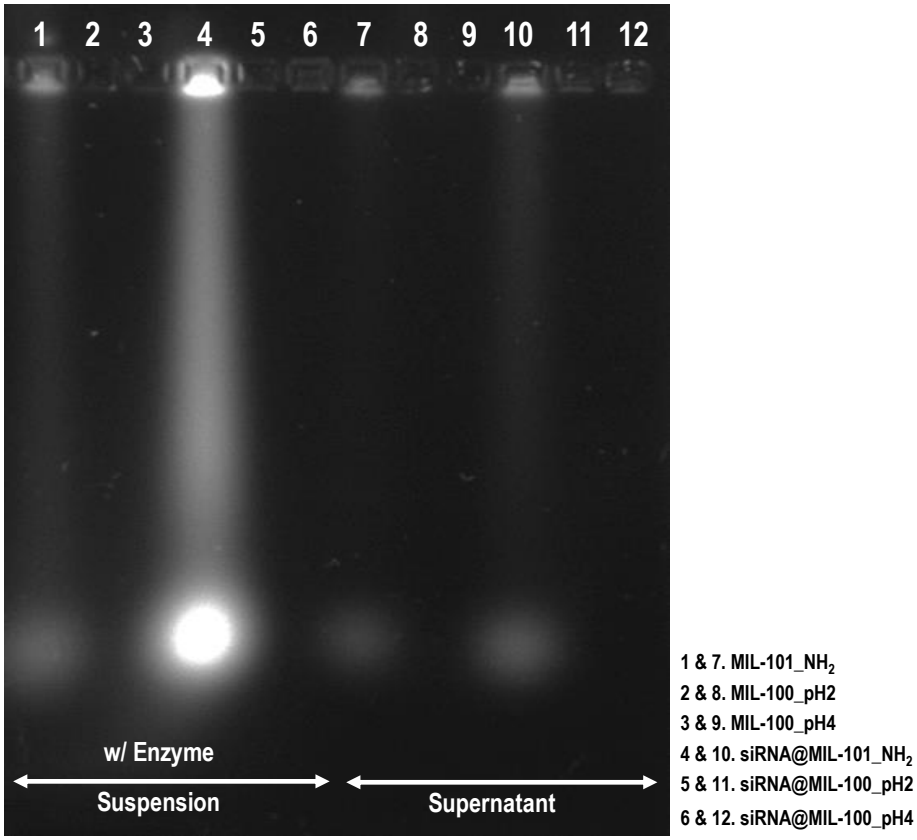

Figure S4. Agarose gel (1%) stained with SYBR® Gold for the enzyme degradation protection analysis of different systems: MOF & siRNA@MOF suspensions with their supernatants.

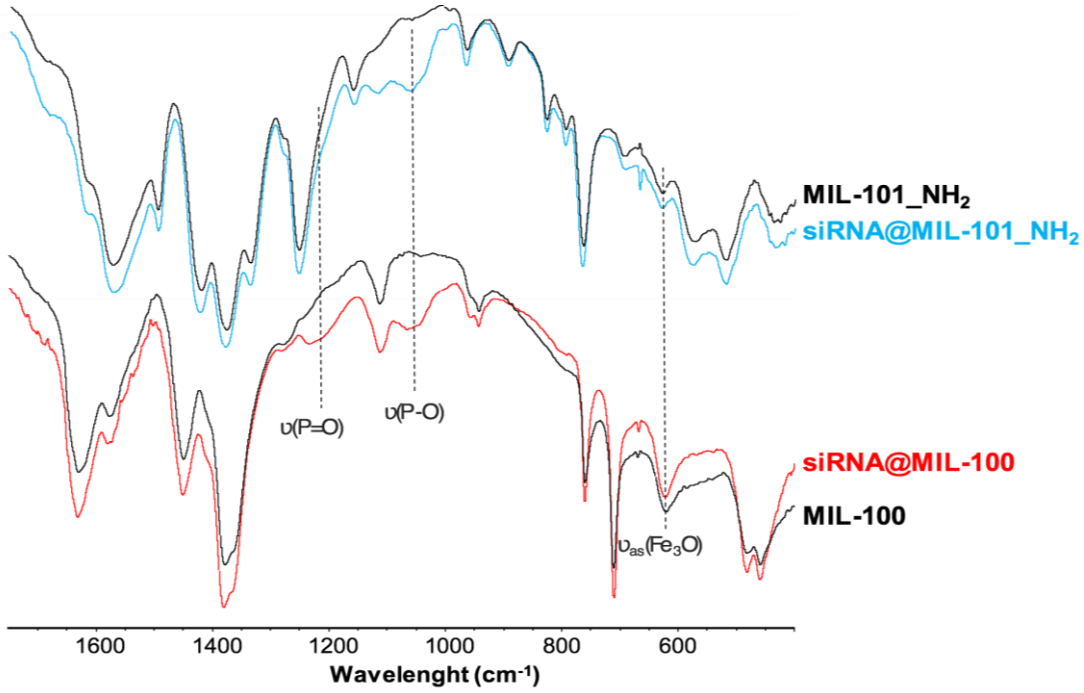

Figure S5. FTIR spectra of MIL-100 and MIL-101_NH_2 NPs before and after siRNA encapsulation at pH 4.

Table S1. Overview of particle size and ζ-potential of MIL-100 (A) and MIL-101_NH_2 (B) NPs in different media (aqueous solution, PBS-FBS and DMEM) at 37°C before and after siRNA association.

A)	Media	MIL-100		siRNA@MIL-100	
		pH=2	pH=4	pH=2	pH=4
Size (nm) (PdI)	H_2O	154 ± 43 (0.2)	195 ± 48 (0.2)	172 ± 25 (0.4)	191 ± 34 (0.4)
	PBS-FBS	167 ± 23 (0.3)	209 ± 62 (0.3)	202 ± 78 (0.4)	366 ± 60 (0.4)
	DMEM	172 ± 74 (0.3)	170 ± 69 (0.3)	277 ± 34 (0.5)	253 ± 42 (0.5)
ξ-potential (mV)	H_2O	+9 ± 1	-21 ± 1	-4 ± 1	-14 ± 2
	PBS-FBS	+10 ± 0	+3 ± 0	+23 ± 3	+4 ± 2
	DMEM	+11 ± 1	-11 ± 1	-1 ± 1	-6 ± 1

B)	Media	MIL-101_NH_2	siRNA@MIL-101_NH_2
		pH=4	pH=4
Size (nm) (PdI)	H_2O	289 ± 2 (0.2)	240 ± 52 (0.3)
	PBS-FBS	422 ± 7 (0.5)	344 ± 77 (0.5)
	DMEM	287 ± 11 (0.4)	308 ± 96 (0.4)
ξ-potential (mV)	H_2O	+12 ± 1	-13 ± 0
	PBS-FBS	+7 ± 0	+8 ± 1
	DMEM	+8 ± 1	+5 ± 1

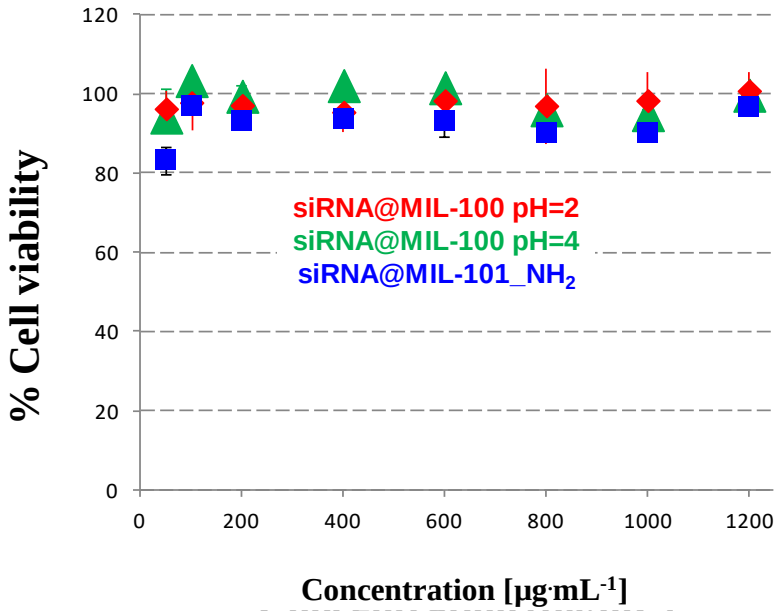

Figure S6. Cell viability of SW480 cell line after 24 h incubation with siRNA@MIL-100 (pH=2; in red and 4; in green) and siRNA@MIL-101_NH_2 NPs (in blue). Note that the shown data correspond for each concentration to the average of triplicates obtained in two independent experiments (*n*=6). The vertical error bars drawn in the diagram indicate the range of fluctuations from which the standard deviations were calculated.

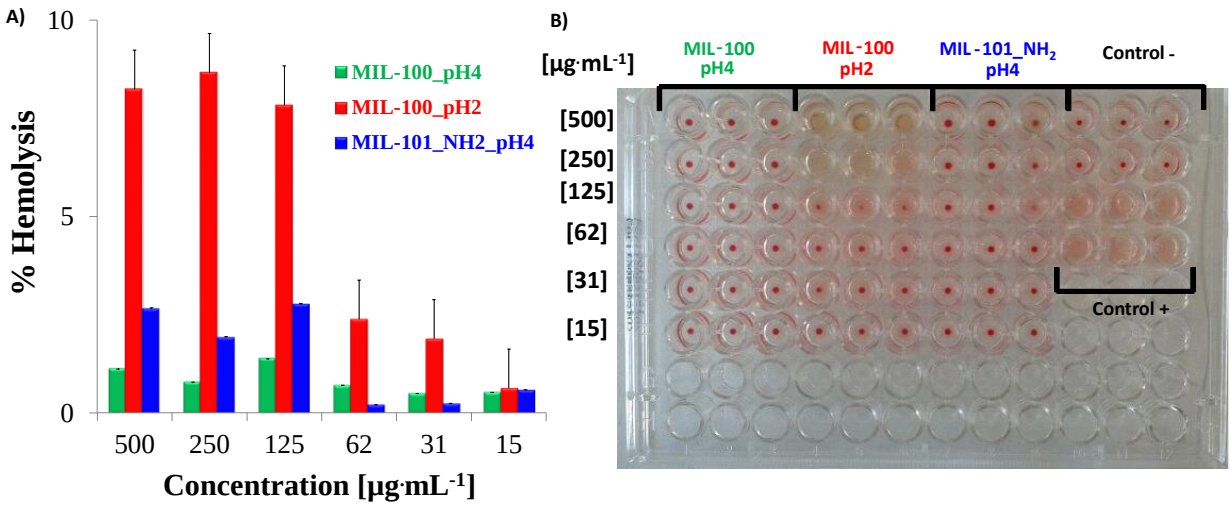

Figure S7. A) Percentage of released haemoglobin after a 15 min incubation of human blood with different concentrations (from 500 to 15 µg.mL^{-1}) of the siRNA_MIL-100 (pH =2 in red or pH = 4 in green) and siRNA_MIL-101_NH_2 (in blue). B) Image of the hemolysis assays of each NP concentration after 15 min of incubation.

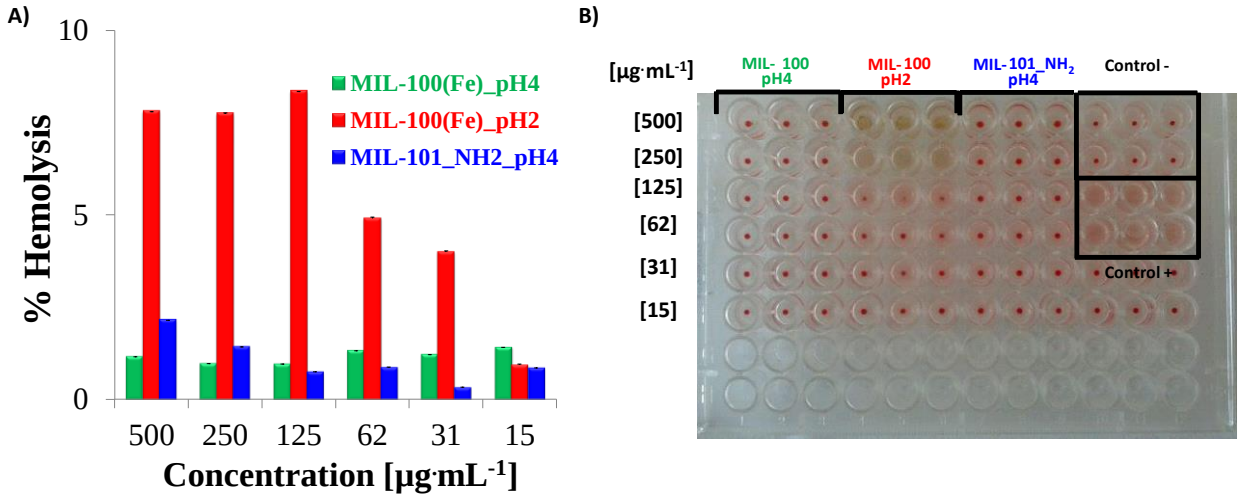

Figure S8. A) Percentage of released haemoglobin after a 1 h incubation of human blood with different concentrations (from 500 to 15 µg.mL^{-1}) of the siRNA@MIL-100 (pH =2 in red or pH = 4 in green) and siRNA@MIL-101_NH_2 (in blue). B) Image of the hemolysis assays of each NP concentration after 1h incubation.

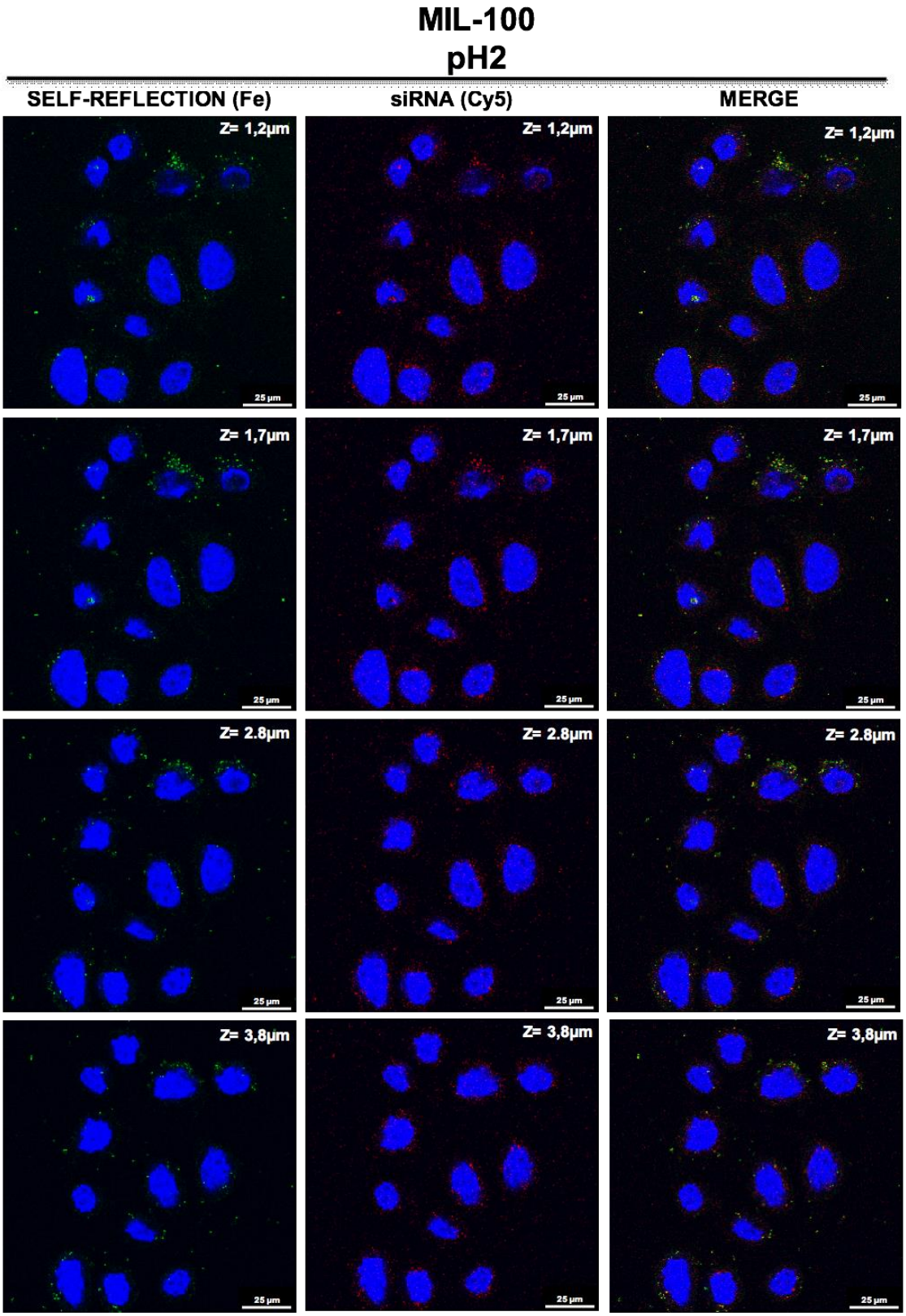

Figure S9. Confocal microscopy images at different depths in the Z-axis of SW480 cell line after 4h of incubation with Cy5-siRNA@MIL-100 (pH=2). MIL-100 NPs, tracked by the iron self-reflection signal, Cy5-labelled siRNA and nucleus, stained with DAPI, are visible in green, red and blue, respectively. The scale bar corresponds to 25 µm. All the images were taken at 63X.

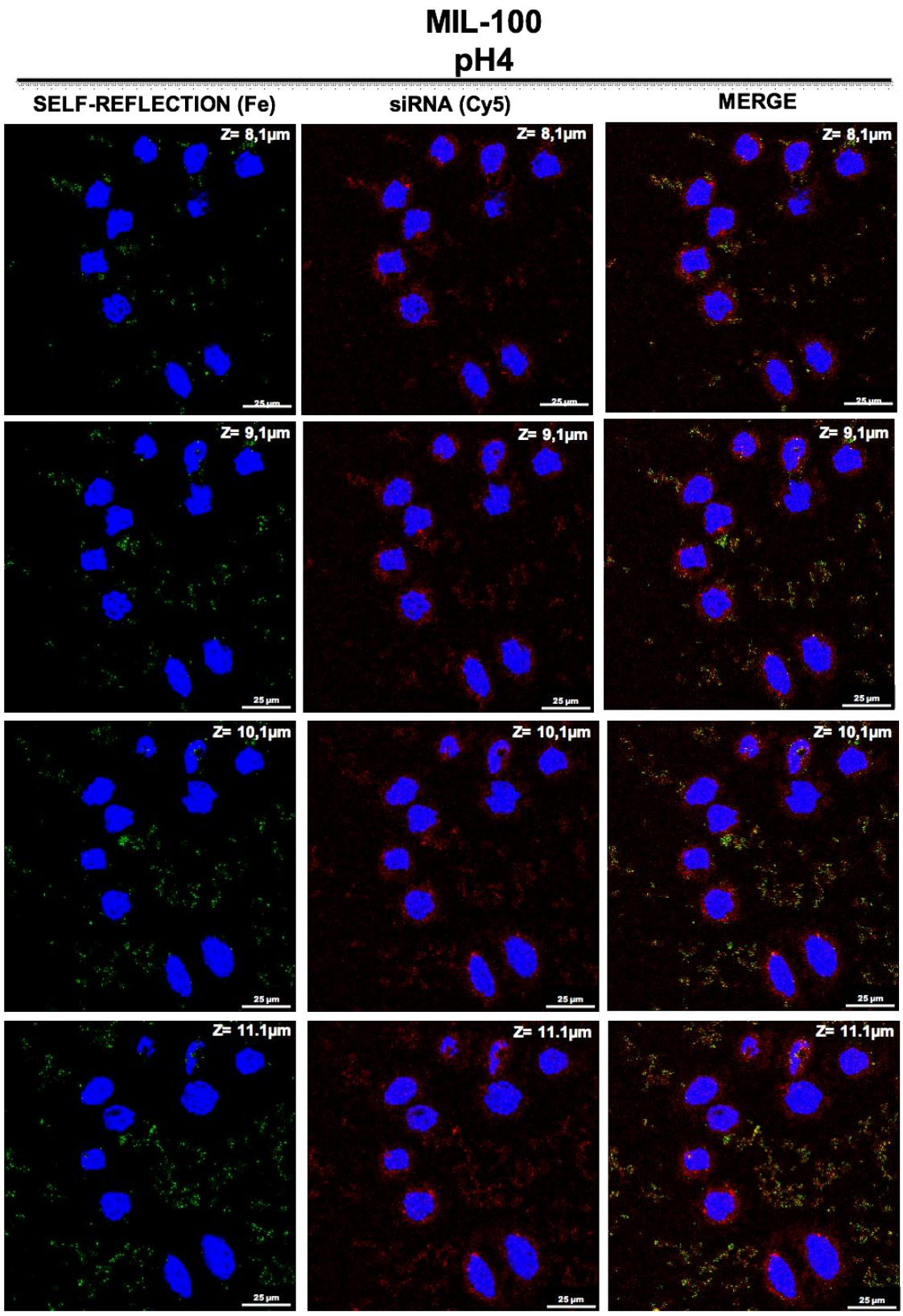

Figure S10. Confocal microscopy images at different depths in the Z-axis of SW480 cell line after 4 h of incubation with Cy5-siRNA@MIL-100 (pH=4). MIL-100 NPs, tracked by the iron self-reflection signal, Cy5-labelled siRNA and nucleus, stained with DAPI, are visible in green, red and blue, respectively. The scale bar corresponds to 25 µm. All the images were taken at 63X.

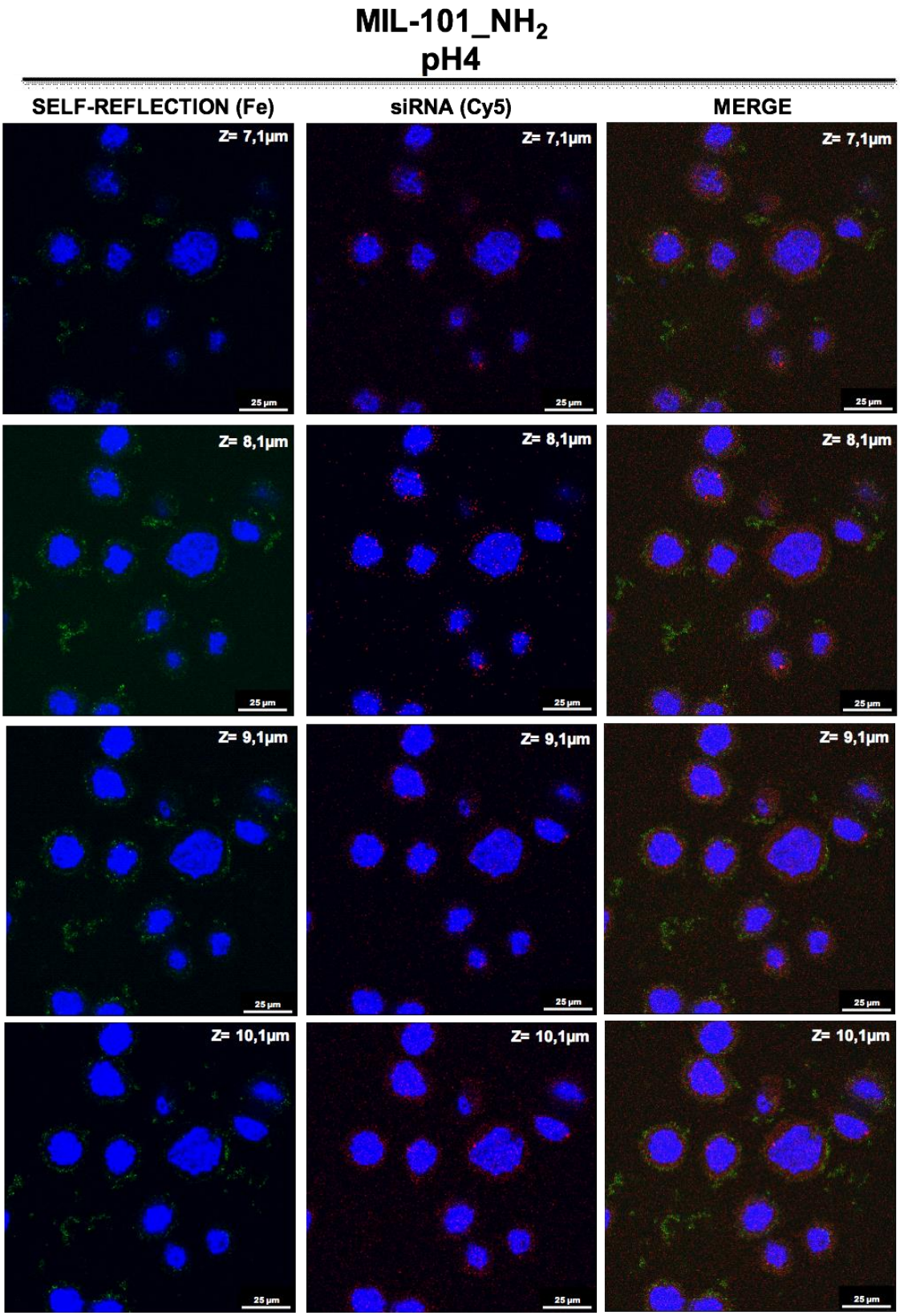

Figure S11. Confocal microscopy images at different depths in the Z-axis of SW480 cell line after 4 h incubation with Cy5-siRNA@MIL-101_NH_2 (pH=4). MIL-101_NH_2 NPs, tracked by the iron self-reflection signal, Cy5-labelled siRNA and nucleus, stained with DAPI, are visible in green, red and blue, respectively. The scale bar corresponds to 25 µm. All the images were taken at 63X.

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