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Supporting Information

Efficent γ-Amino-Proline-derived Cell Penetrating Peptide-Superparamagnetic Iron Oxide Nanoparticle Conjugates via Aniline-Catalyzed Oxime Chemistry as Bimodal Imaging Nanoagents

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Experimental section.

General materials and methods.

All reagents and solvents were commercial products purchased from commercial sources and used as received. Nanomag®-D–spio (NH_2 surface, 20 nm; 5 mg/ml solid content and 2.4 mg/mL iron concentration) were obtained from Micromod Partikeltechnologie GmbH, Germany. Protected amino acids were obtained from Neosystem (Strasbourg, France) and MBHA resin (0.63 mmol/g) was supplied by Calbiochem-Novabiochem AG. Diisopropylcarbodiimide (DIC) was obtained from Fluka Chemika (Buchs, Switzerland) and HOBt from Iris Biotech (Marktredwitz, Germany). 5(6)-carboxyfluorescein (CF) was obtained from Aldrich (Milwuakee, WI). Solvents for peptide synthesis and RP-HPLC equipment were obtained from Scharlau (Barcelona, Spain). Trifluoroacetic acid was supplied by KaliChemie (Bad

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Wimpfen, Germany). Other chemicals were obtained from Aldrich (Milwaukee, WI) and were of the highest commercially available purity. All commercial reagents and solvents were used as received. HF was obtained from Air Products and Chemicals, Inc. (Allentown, Canada), and related equipment was obtained from Peptide Institute Inc., Minoh, Osaka, Japan. Analytical RP-HPLC was performed using Waters (Milford, MA) chromatography systems with reverse-phase Symmetry C_{18} (150 × 43.9 mm) 3.5 μm columns with UV detection at 220 nm. Semipreparative RP-HPLC was performed on a Waters (Milford, MA) chromatography system using Symmetry C_8 (3 × 10 cm, 5 μm) columns. Compounds were detected by UV absorption at 220 nm. Milli-Q water with a resistance of more than 18.2 MΩ/cm was provided by a Millipore Milli-Q filtering system with filtration trough a 0.22 μm Millipak filter. TLC analysis was conducted on TLC-plastic sheets 60 F_{254} (Merck) with detection by UV absorption where applicable and/or by staining with a solution of ammonium molybdate and/or a solution of ninhydrin, followed by charring at ~ 150ºC. Column chromatography was performed using silica gel (0.063-0.200 mm particle size, 70-230 mesh). Mass spectra were recorded on a MALDI Voyager DE RP time-of-flight (TOF) spectrometer (Applied Biosystems, Framingham) or by LC-MS analysis with reverse-phase Symmetry C_{18} (150 × 43.9 mm) 3.5 μm columns with UV detection at 220 nm. 2,5-dihydroxybenzoic acid (DHB) was used as a matrix and was purchased from Aldrich. ^{1}H-NMR spectra were acquired with a Varian 400 NMR spectrometer (400 MHz). Chemical shifts are reported in ppm with tetramethylsilane (TMS) as an internal reference. Abbreviations used are s = singlet, d = doublet, dd = doublet of doublets, m = multiplet, br = broad.

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Solid-Phase Peptide Synthesis (SPPS). Peptide syntheses were performed manually in a polypropylene syringe fitted with a polyethylene porous disc. Solvents and soluble reagents were removed by suction. Washings between deprotection, coupling and subsequent deprotection steps were carried out with DMF (5 ×), DCM (5 ×) using the right amount of solvent to properly swell the resin for each wash. A peptide containing the translocation peptide sequence consisting of residues 48-57 of the TAT protein (Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-NH_2) was synthesized by Fmoc solid phase chemistry using a Rink amide MBHA resin (0.25 mmol, 0.59 mmol/g, 424 mg). The resin was initially washed with DCM (2 ×) followed by further washing with DMF (2 ×). The Fmoc group was removed as described in the general procedure. The amino acids were coupled by adding a pre-activated mixture of the appropriate Fmoc-protected amino acid: Fmoc-Arg(Pbf), Fmoc-Gln(Trt), Fmoc-Lys(Boc), Fmoc-Gly (4 equiv.), DIC (4 equiv.)/HOBt (4 equiv.) and DIPEA (8 equiv.) in DMF/DCM 1/1 v/v (4 ml) for 2 h or overnight. All coupling steps were followed by Kaiser test on the resin to indicate presence (deprotection) or absence (coupling) of free amino groups. After each deprotection and coupling step, the resin was drained and washed with DMF (4 ×) and DCM (4 ×). Fmoc-Lys(Mtt) amino acid, 5(6)-carboxyfluorescein (CF) and the Boc-NH-OCH_2COOH were coupled to the peptide anchored to the resin as described in the general procedures on 100 mg of resin. The solid support-attached TAT-derived peptide was cleaved off the resin as described in the general conditions to afford crude 24.6 mg of product as orange powder. When needed, crude peptide was purified by semi-preparative RP-HPLC using standard conditions. The product was

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characterized by ESI-MS and MALDI, expected mass 1954.06 m/z: found 1955.38 m/z for [(M+H)+].

For the synthesis of the cis-γ-amino-L-proline-derived peptide **a** we employed a combined Fmoc/Boc solid phase strategy on MBHA resin as followed.[1] 300 mg of MBHA resin were used. Firstly, two blocks of Fmoc-8-amine-3,6-dioxaoctanoic acid were coupled to the resin using standard coupling method. Then, alternate incorporations of Fmoc-Amp(Boc)-OH (3 equiv.) and Fmoc-Amp(Alloc)-OH (3 equiv.) were carried out with DIC (3 equiv.) and HOBt (3 equiv.) in DMF for 2 h. The resin was washed with DMF (5 ×) and DCM (5 ×) after each coupling. Couplings were monitored by the Kaiser test. Once the alternate Boc/Alloc protected skeleton had been built, the N^{α}-Boc protecting group was removed. Alkylation of the amino group was performed by on-resin reductive amination using phenylacetaldehyde (5 equiv. for each amine) in 1% HOAc in DMF for 30 min and then treating the resin with $NaBH_3CN$ (5 equiv. each amine) in MeOH for 2h. After that, the resin was washed with DMF (5 x) and DCM (5 x). Alkylation was monitored by chloranil test. After removal of the N^{α}-Alloc protecting group, the corresponding alkylation with isovaleraldehyde was performed as described above. When needed, the crude product was purified using a linear gradient (from 0 to 50% of MeCN in 25 min) of MeCN (containing 0.1% of TFA) and H_2O (containing 0.1% of TFA). The product was characterized by ESI-MS and MALDI, expected mass 2119.7 m/z: found 2121.0 m/z for $[(M+H)^+]$ and 2142.0 m/z for $[(M+Na)^+]$. ESI mass found m/z: 1060.9 (z = 2), 707.4 (z = 3), 530.8 (z = 4).

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Later, Fmoc-Lys(Mtt) amino acid, 5(6)-carboxyfluorescein (CF) and the Boc-NH-OCH_2COOH were coupled to the peptide anchored to the resin as described in the general procedures.

General Procedures:

Fmoc Group Removal. (i) DMF (5 × 1 min); (ii) piperidine/DMF (2:8) (1 × 1 min + 2 × 10 min); (iii) DMF (5 × 1 min).

Boc Group Removal. (i) DCM (5 × 1 min); (ii) TFA/DCM (4:6) (1 × 1 min + 1 × 25 min); (iii) DCM (5 × 1 min); (iv) DIPEA/DCM (5:95) (3 × 3 min); (v) DCM (5 × 1 min).

Alloc Group Removal. (i) DCM (5 × 1 min); (ii) $Pd(PPh_3)_3$/PhSiH (0.1:10) (2 × 10 min in DCM); (iii) DCM (5 × 1 min).

Fmoc-Lys(Mtt) Coupling.

Coupling of the Fmoc-Lys(Mtt) was performed by adding a pre-activated solution of Fmoc-protected amino acid (4 equiv.), DIC (4 equiv.) and DIPEA (8 equiv.) in DMF/DCM 1/1 v/v (4 ml) to the peptide linked to the resin (in general 100 mg of resin were used). After overnight coupling, the resin was washed with DMF (5 × 1 min).

Mtt Removal.

3% TFA in DCM for (i) 10 min, (ii) and (iii) 5 min. Wash with DCM (5 × 1 min).

5(6)-Carboxyfluorescein (CF) Coupling.

CF (2 equiv.) was coupled to the N^{ε} group using DIC/HOBt/DIPEA (2/2/4 equiv.) overnight. After that time, resins were filtered and washed several times with DMF. In some cases re-coupling was needed and it was carried out using the same

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conditions. To avoid over incorporation of CF, two 30-min treatments with 20% piperidine-DMF were carried out before the cleavage of the peptide from the resin.[2]

Boc-NH-OCH_2COOH Coupling.

Boc-NH-OCH_2COOH (2 equiv.) was coupled to the N^{ε} group using DIC/HOBt/DIPEA (2/2/4 equiv.) overnight. After that time, resins were filtered and washed several times with DMF. In some cases re-coupling was needed and it was carried out using the same conditions.

Cleavage with TFA.

Peptide was cleaved off the Rink amide MBHA resin with TFA/water/TRIS/m-cresol (90:5:2.5:2.5 v/v/v/v) for 2 h and washed with TFA. The crude product was precipitated with cold Et_2O. The precipitate was centrifuged, washed with an additional amount of Et_2O (2 ×), centrifuged, re-suspended in water/AcCN 1/1 v/v and lyophilized.

Acidolytic cleavage with HF. The MBHA peptide resin was washed with MeOH (3 ×), dried and treated with HF in the presence of 10% anisole for 2 h at 0 ºC. Peptides were precipitated with cold anhydrous methyl tert-butyl ether (MTBE), dissolved in HOAc 10% in H_2O and then lyophilized.

Synthesis of the *N*-Hydroxysuccinimide ester of 4-formylbenzoic acid.

4-formylbenzoic acid (150 mg, 2.2 mmol), *N*-hydroxysuccinimide (1.2 equiv., 2.2 mmol, 138 mg), and EDC (2 equiv., 2.3 mmol, 383 mg) were dissolved in 50 mL of anhydrous DCM. Reaction mixture was stirred at RT overnight and TLC (5% MeOH in DCM) revealed completeness of the reaction. The solvent was removed under reduced pressure. Crude product was purified by silica chromatography (DCM

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to 10% v/v MeOH in DCM) to provide 494 mg of the product as a white solid (95% of yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 10.2 (s, 1H, CO*H*), 8.0 (d, 2H, Ar), 8.3 (d, 2H, Ar), 2.9 (br s, 4H, CH_2CO). MS calculated for [M + H]+ ($C_{12}H_9NO_5$): 248.05 m/z, found: (ES+) 248.24 m/z.

Synthesis of SPION-CHO

Amine functionalized cross-linked dextran coated Fe_3O_4 nanoparticles (**SPION-NH_2**) were used as received from Micromod. 2 mL of nanoparticle solution were diluted into 2 mL of 25 mM citrate buffer at pH 8, and the mixture was added to the NHS ester of 4-formilbenzoc acid (4 mg, 0.016 mmol), followed by addition of diisopropylethylamine (DIPEA) (10 µL).[3] The reaction mixture was rotated overnight at room temperature and then dialyzed against water for 3 days with several water changes. Collected solution was diluted with water to 10 mL final volume and used as such in the following reactions.

Protocol for the aniline-catalyzed oxime chemistry.

2 mg of aminooxyacetyl-derived peptides **a-c** were solubilized in 1 µL DMSO and dispersed in 500 µL of 0.1 M Na phosphate (pH 7.5). To this mixture 500 µL of **SPION-CHO** solution were added as well as 9 µL of aniline (100 mM).[4] Reactions were carried out in a test tube under rotation overnight at room temperature in the dark. Samples were dialyzed using a 3.5 KDa molecular weight cutoff (MWCO) SpectraPor regenerated cellulose against milliQ water or PBS.

Fluorescence spectroscopy.

Fluorescent spectra were measured with a Varian Cary Eclipse Fluorescence spectrometer. Measurements were performed on dialyzed sample using 1 mm path

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cuvette for measuring fluorescence. Emission spectra were recorded from 430 nm to 620 nm by exciting at 492 nm. In general both excitation and emission slits were of 10 or 5 nm depending of the fluorescence intensity. Cuvettes were cleaned with water and ethanol and dried with a N_2 flow.

HRMAS-NMR.[5]

NMR experiments were carried out on a Bruker DMX 500 (11.7 T) equipped with a HRMAS ^{1}H-^{13}C indirect detection probe with gradients on the magic angle. MAS experiments were performed at spinning rates of up to 8 kHz (15 kHz maximum MAS rotation available) using a 50 µL zirconia rotor. In general, 1-2 mg of lyophilized nanoconjugate powders were dispersed in 60 µL of deuterated DMSO. Proton spectra were obtained using 1024-2048 scans for each experiment. The sample temperature was kept constant at 298 K.

TEM.

For transmission electron microscopy (TEM) analysis, a drop of each sample was placed on a carbon-coated copper grid and dried on air. TEM images were obtained by a Jeol J1010 electron microscope (Jeol, Japan) equipped with a Megaview III CCD camera.

IR.

IR spectroscopy on lyophilized samples were obtained using a ATR diamond Thermo IZ10 with DTGS detector, KBr divisor and Glowbar source or a ABB FTLA2000 Spectra Tech. microscope, with MCT detector.

UV determination of 5(6)-Carboxyfluorescein (CF).[6]

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5(6)-Carboxyfluorescein (CF) concentration was determined spectrophotometrically by measuring each sample after dialysis as such or by diluting an aliquot of it in PBS in order to get absorbance values ranging between 1 and 0.1. In general, the absorbance maxima of the fluorochrome (in this case around 500 nm) multiplied by the dilution factor and divided by the extinction coefficient of the dye (ε = 81000 M^{-1} cm^{-1} for CF in PBS) give the fluorochrome concentration. In the majority of the cases, concentrations were 1.5-2 μM of dye before dilution.

Determination of Fe concentration.[6]

Iron concentration was determined spectrophotometrically after acid dissolution and oxidation. 50-100 μL of sample were mixed with 6 M hydrochloric acid containing 3% hydrogen peroxide to 1 mL of total volume and let stand for 1 h at room temperature. Optical density was measured at 410 nm. Standard solutions containing 0.01–0.1 mg/mL of Fe were used for the standard line and were prepared by proper dilution of the commercially available SPION solution (iron content: 2.4 mg/mL) in 6 M HCl containing 3% hydrogen peroxide. In the majority of the cases, concentrations were 2-3 mM of iron before dilution.

General procedure for Cell Culture.

HeLa cells were maintained in DMEM (1000 mg/mL glucose) culture medium (Biological Industries) containing 10% fetal calf serum (FCS), 2 mM glutamine, 50 u/mL penicillin, and 0.05 g/mL streptomycin. For all experiments, exponentially growing cells were detached from the culture flasks using a trypsin-0.25% EDTA solution, and the cell suspension was seeded onto 96-well (Nunclon surface, Nunc International, Napvrille, USA) or 35 mm plates (Nunclon surface, Nunc

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International, Napvrille, USA) at 21.4 x 10^3 cells/cm^2. Experiments were carried out 24 h later, at a confluence level of approximately 60-70%. Nanoconjugate solutions in PBS were diluted with the cell culture medium to a final concentration of CF of 1 μM. Non-adherent cells were washed away and attached cells were incubated with the SPION-peptide samples in DMEM at 37 ºC in CO_2 atmosphere (5%).

Uptake Measurements by Flow Cytometry. The cellular uptake of nanoconjugates was first analyzed by flow cytometry. HeLa cells were seeded onto 35 mm plates at a concentration of 21.4 x 10^3 cells/cm^2. After 24 hours, cells were incubated with nanoconjugate solutions in PBS diluted with the cell culture medium to a final concentration of CF of 1 μM. After 6 h, cells were washed three times with PBS, detached with tripsine-EDTA 0.25%, centrifuged at 1000 x *g*, washed again and measured. In order to remove any possible fluorescence of CF-peptide nanoconjugates bound to the plasma membrane, the pH of the solution was brought down to six by the addition of 1N HCl (at pH 6, extracellular fluorescence of CF is quenched without altering cell viability).[7] Supplementary experiments with addition of propidium iodide (PI) were performed using the same incubation conditions as described above. Cells stained with PI were excluded from further analysis. Obtained results showed no substantial difference from non-treated cells.

Fluorescence analysis was performed with an Epics XL flow cytometer (Coulter). Triplicates of each sample were performed for each condition and results from independent experiments were normalized by subtraction of auto-fluorescence control value from each value and dividing by the fluorescence value obtained from the CF control under the same experimental conditions.

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Quantification of intracellular iron concentration.

HeLa cells were seeded onto 35 mm plates at a concentration of 21.4 x 10^3 cells/cm^2. After 24 hours, cells were incubated with nanoconjugate solutions in PBS diluted with the cell culture medium to a final concentration of CF of 1 μM. After 6 h, cells were washed three times with PBS, detached with tripsine-EDTA 0.25%, centrifuged at 1000 x *g*, washed again. Aliquots (200 μL) were disperse in 1.5 mL of HNO_3 and 0.5 mL of H_2O_2. After overnight digestion at 90 °C, samples were diluted in 10 mL of ultrapure H_2O and weighted in order to obtain a density value of the final samples. Iron concentration was measured by atomic absorption spectroscopy (AAS). In the case of the solution containing conjugate **1a** (d = 1.05657), 3.6 μg/mL of iron were found, while the blank solution (d = 1.05148) contained 1.4 μg/mL of iron.

Confocal Microscopy.

HeLa cells (21.4 x 10^3 cells/cm^2) were plated onto glass bottom Lab-Tek chambers for live-cell imaging and cultured overnight. The medium was discarded and cells were washed with PBS followed by treatment with 1 μM concentration of CF of each nanoconjugate sample in complete culture medium for 6 h under routine culture conditions. After incubation time, medium was removed and cells were washed with PBS and fixed with MeOH at -20 ºC. After fixation, MeOH was removed and cells were washed again with PBS, distilled water and then were mounted with Mowiol. The cells were observed with a Leica SP2 confocal microscope using a 60X/1.4 NA plan-apochromatic objective. A 488 nm line of

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argon laser was used to excite CF and its emision was detected in a range of 515-530 nm. The conditions used were kept constant for all imaging experiments.

MRI.

Phantom preparation.

HeLa cells were seeded onto 35 mm plates at a concentration of 21.4 x 10^3 cells/cm^2. After 24 hours, cells were incubated with the different nanoconjugate samples, containing 250 μM of Fe, for 4 h at 37 ºC and 5% CO_2; control cells were incubated in the absence of nanoparticles. Cells were then washed three times with PBS, detached with tripsine-EDTA 0.25 %, centrifuged at 1000 x g and washed once more by resuspension in 1 mL of medium. Cells were counted and solubilized in PBS in order to reach a final number of 250 x 10^3 cells/mL. 1% agarose gel phantoms were prepared by mixing 250 μL of cell samples in PBS with 250 μL of hot (100 °C) 2 wt% agarose solution in de-mineralized water in a 500 μL plastic text tube. Samples were immediately vortexed to obtain an homogeneous mixture, then allowed to cool to 25°C for gelation and stored at 4 ºC until measurements. Non-homogeneous preparations (e.g. due to air bubble formation) were not used for MRI analysis.

MRI Measurements.

MRI images of the phantoms were acquired in sagittal and transversal planes. These studies were carried out on a 7 T horizontal magnet (BioSpec 70/30 USR; Bruker BioSpin, Ettlingen, Germany) equipped with actively shielded gradients (B-GA12 gradient coil inserted into a B-GA20S gradient system), which is part of the CIBER-BBN Platform of Biomedical Applications of Nuclear Magnetic Resonance

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at the SeRMN of the UAB. Experimental parameters for the T2-weighted spin-echo images were: rapid acquisition with relaxation enhancement (RARE) sequence with repetition time (TR) 4,200 ms, effective echo time (TE_{eff}) of 10, 50, 90 and 130 ms (see figure 3C of the main text), rare factor of 16, number of averages (NA) = 4 (except for T2w image with TE_{eff} of 10 ms, where rare factor was 8 and NA = 2), matrix (MTX) 512 x 256, field of view (FOV) 90 x 45 mm (0.176 mm/pixel), slice thickness 4 mm and total acquisition time of 3 min 21 sec.

For T2 measurements a multi-slice multiecho (MSME) sequence was used, with TR of 5,000 ms, echo times between 10 and 500 ms in steps of 10 ms (total = 50 echoes), MTX 256 x 160, FOV of 90 mm x 45 mm (0.352/0.281 mm/pixel), slice thickness 4 mm and total acquisition time of 10 min 10 sec.

Fig 3C of the main text is a composed image from T2-weighted high resolution MR images at half height of the phantom tube length, obtained with different effective echo times.

T2 values were calculated as the media of a duplicate prepared with the same agarose gel. In this case phantoms were totally homogenous. We decide to exclude T2 values obtained during other experiments as we observed variations in case of dishomogeneity of samples or by varying agarose gel preparation. Standard deviations were therefore not not calculated (only n = 2)**.**

A relaxivity value of 16.87 $mm^{-1} \cdot s^{-1}$ for CPP-SPION **1a** was obtained by T2 calculations obtained using a multi-slice multiecho (MSME) sequence at 7 T. For these measurements phantoms containing saline solutions of nanoconjugate **1a** at different iron concentrations (0, 80, 160, 320, 640 μM) were used.

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MTT Cytotoxicity Assay. The viability of HeLa cells in the presence of the SPION-peptide conjugates was tested using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In order to avoid saturation in cell growth after 24 h of conjugates incubation, 7 x 10^3 cells/well were seeded on a 96-well plate (Nange Nunc) for each assay. After 24 hours, the culture medium was discarded and replaced by nanoconjugate solutions in PBS that were diluted with the cell culture medium to a final concentration of 1 μM of CF or 250 μM of Fe. Cells were incubated for 6 h at 37 ºC under 5% CO_2 atmosphere and MTT (0.5 mg/mL) was added 2 h before the end of incubation. After 2 hours of incubation with MTT, the medium was discarded by aspiration and isopropanol was added to dissolve formazan, a dark blue colored crystal observed in the wells. Absorbance was measured at 570 nm in a spectrophotometric Elx800 Universal Microplate Reader (Bio-Tek), 30 minutes after the addition of isopropanol. Cell viability is expressed as an absorbance percent ratio of cells treated with peptide to untreated cells, which were used as a control.

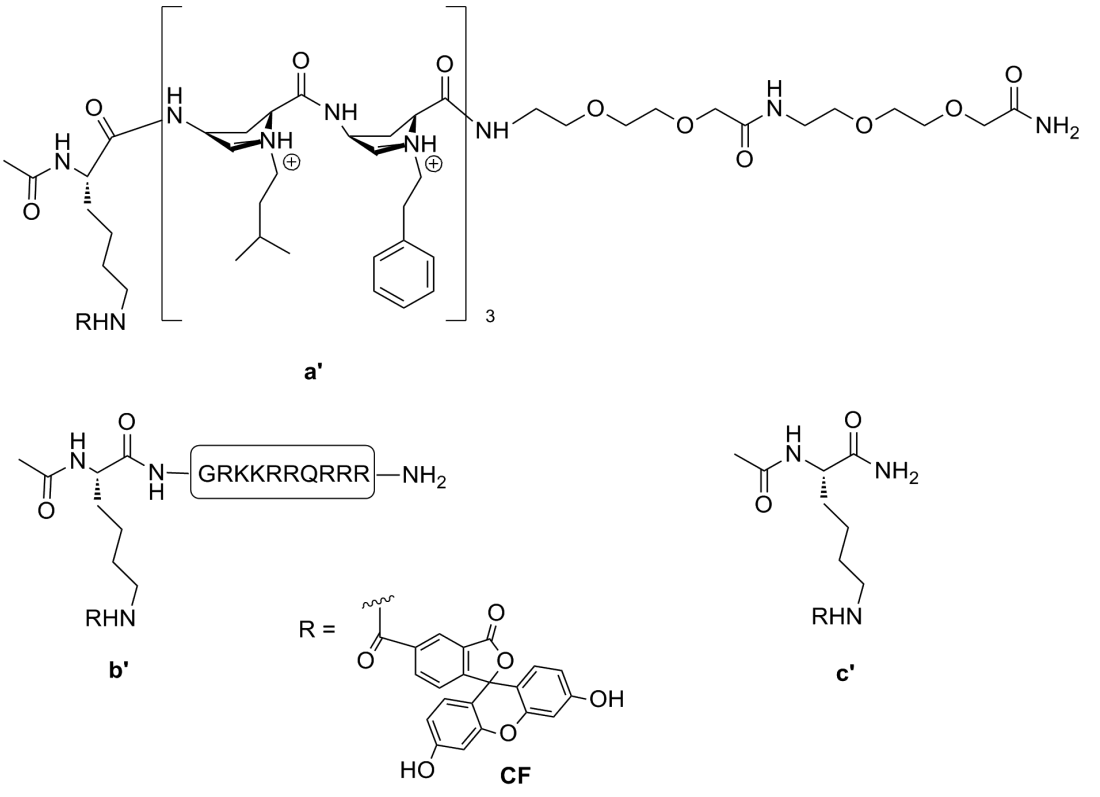
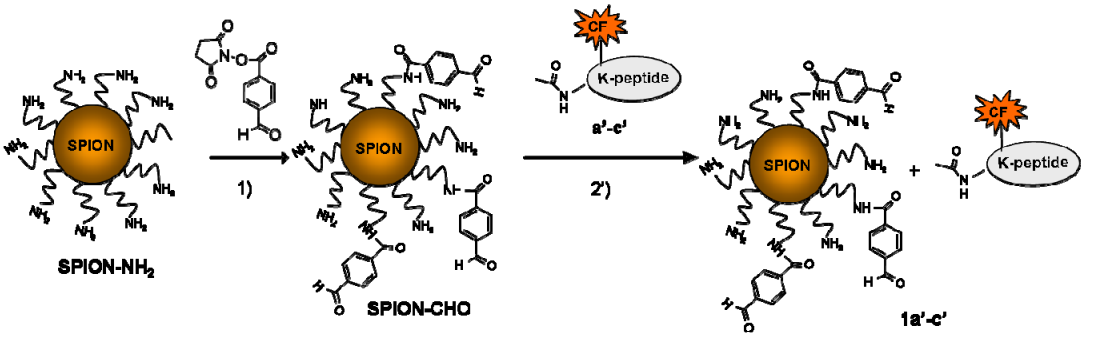


Chart 1S. Molecular structure of acetylated derived peptides **a'-c'**.



Scheme 1. Schematic representation of the synthesis of CCP-SPION conjugates **1a-c**. Reagents and conditions. (1) 4 mg *N*-hydroxysuccinimidyl ester of 4-formylbenzoic acid, 10 µM DIPEA in 0.1 M citrate buffer pH 8. (2') 2 mg of acetylated peptides **a'-c'**, 100 mM aniline in 0.1 M phosphate buffer pH 7.5.

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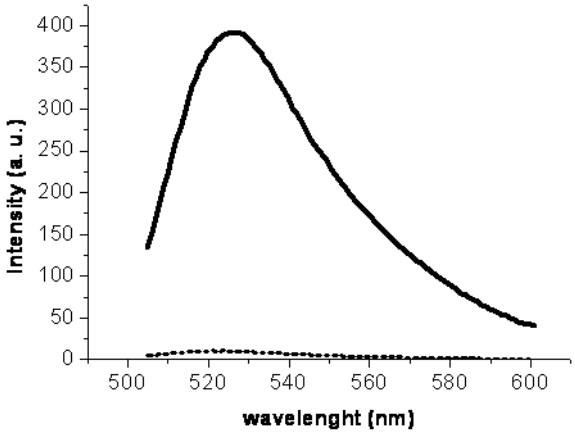

Figure S1. (A) Fluorescence spectra of conjugate (—) **1b** and control (····) **1b'**.

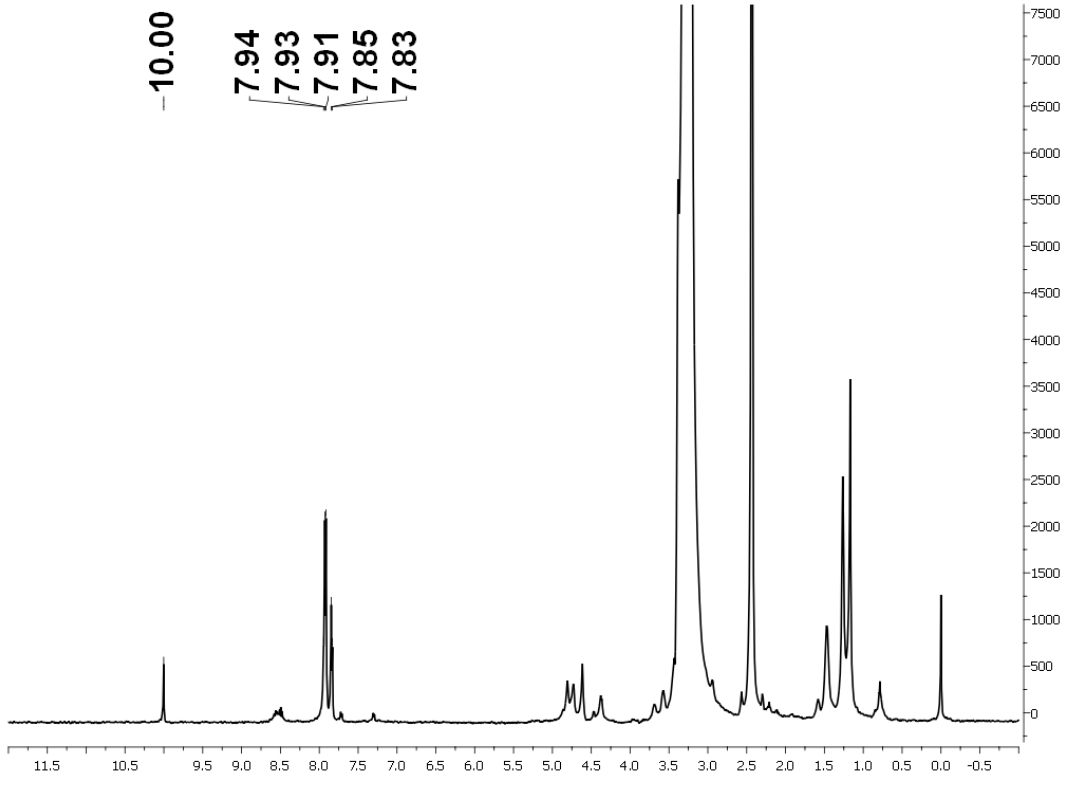

Figure S2. HRMAS-NMR of conjugate **SPION-CHO**.

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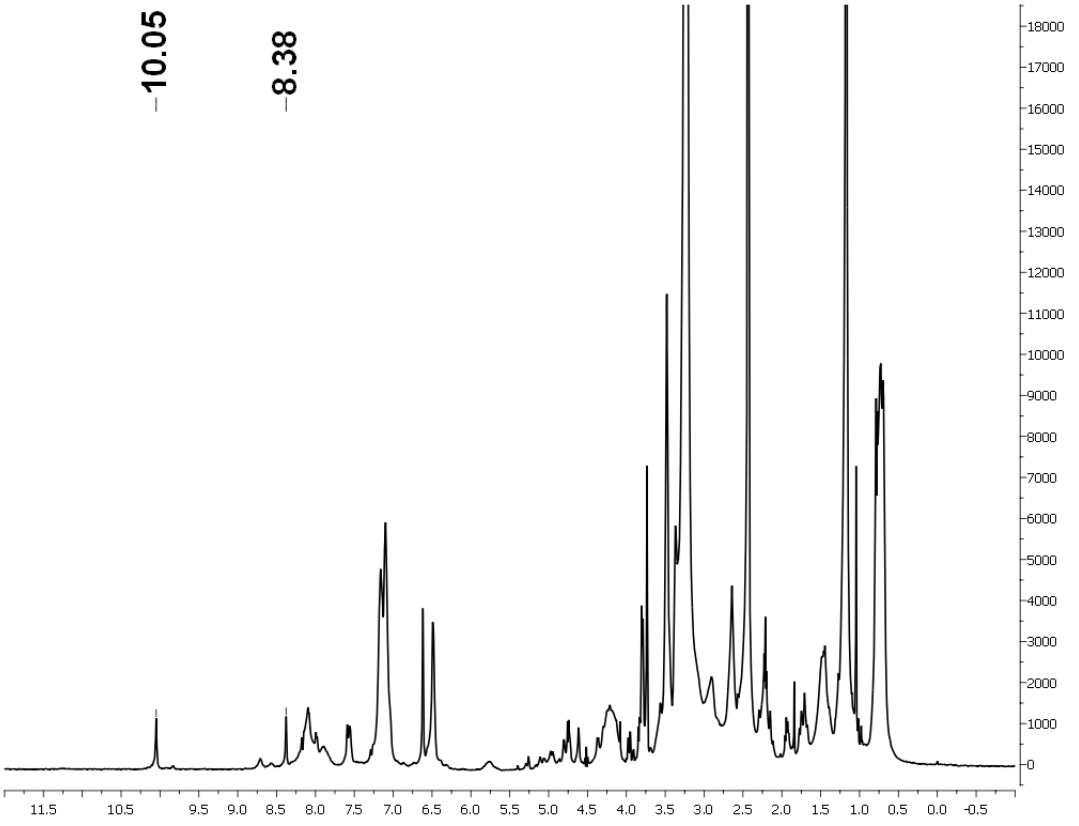

Figure S3. HRMAS-NMR of conjugate **1a**.

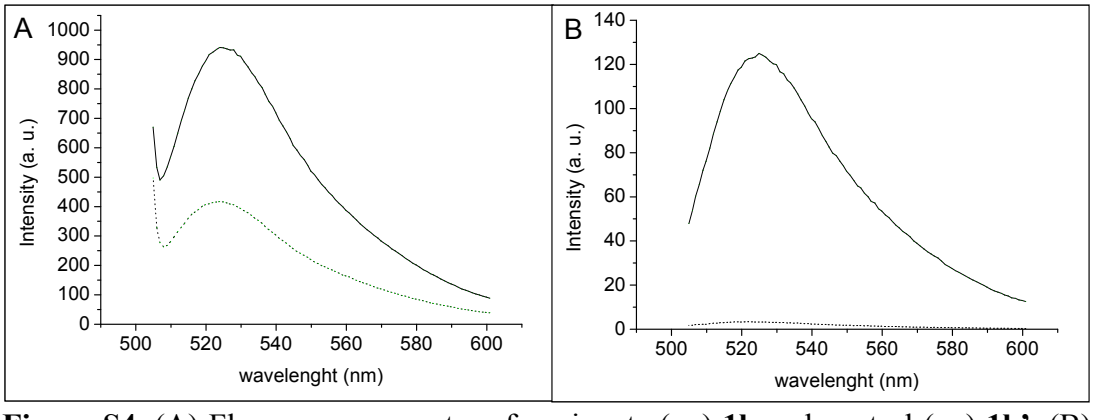

Figure S4. (A) Fluorescence spectra of conjugate (—) **1b** and control (····) **1b'**. (B) Fluorescence spectra of conjugate (—) **1c** and control (····) **1c'**.

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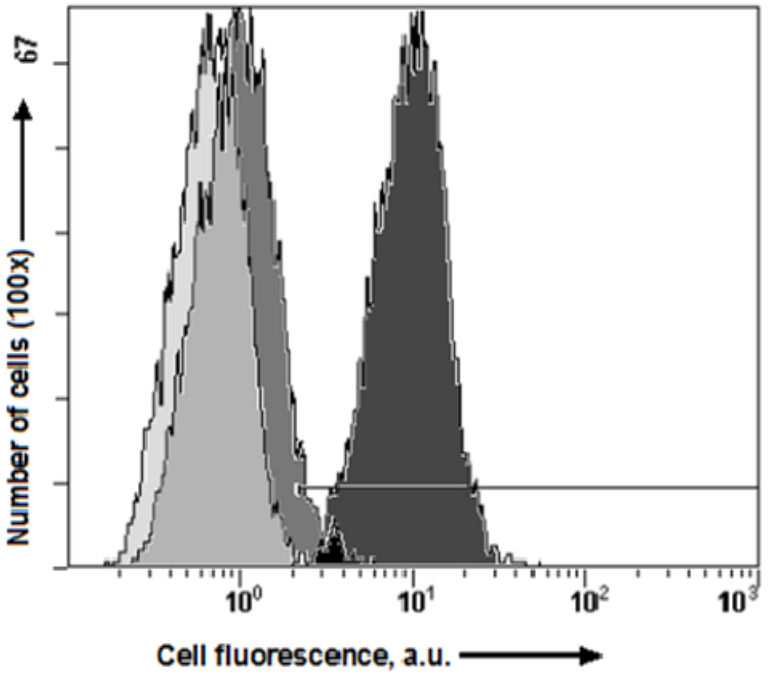

Figure S5. Flow cytometry analysis. HeLa cells were incubated with only medium (light gray), nanoparticle solution **1a** (black) and control nanoparticle solution **1a'** (dark gray) at a final concentration of 1 μM of CF for 6 h.

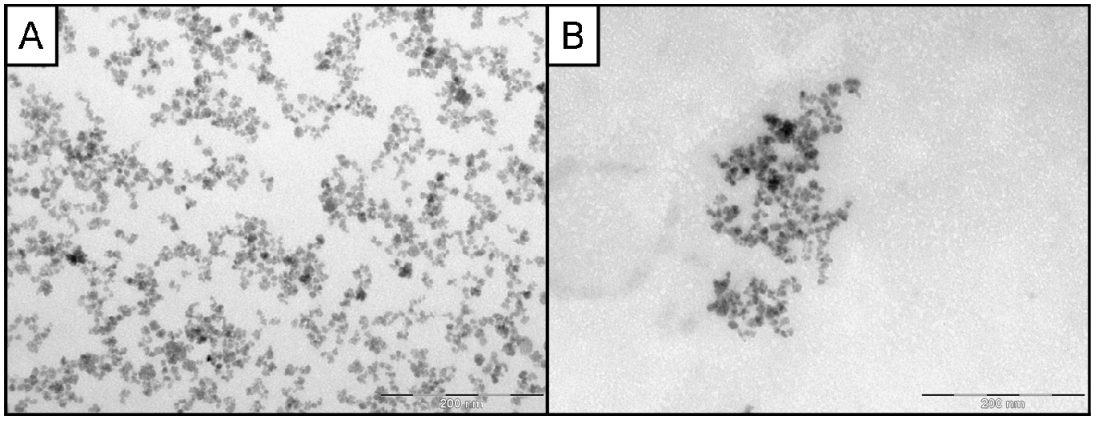

Figure S6. TEM image of nanoparticle solution (A) **SPION-NH_2** and (B) **1a**. Scale bar 200 nm.

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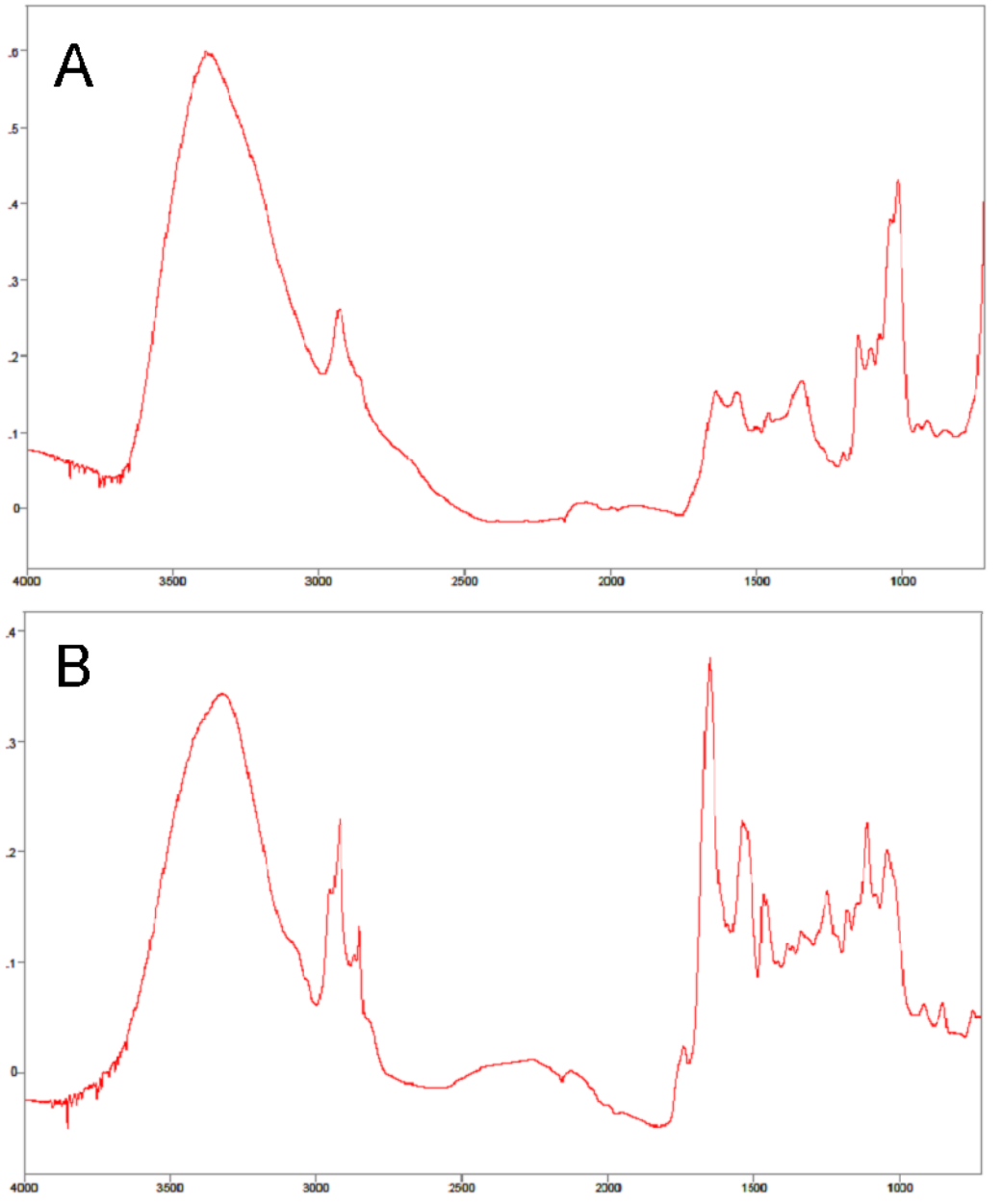

Figure S7. IR spectra of nanoparticle solution (A) **SPION-NH_2** and (B) **1a**.

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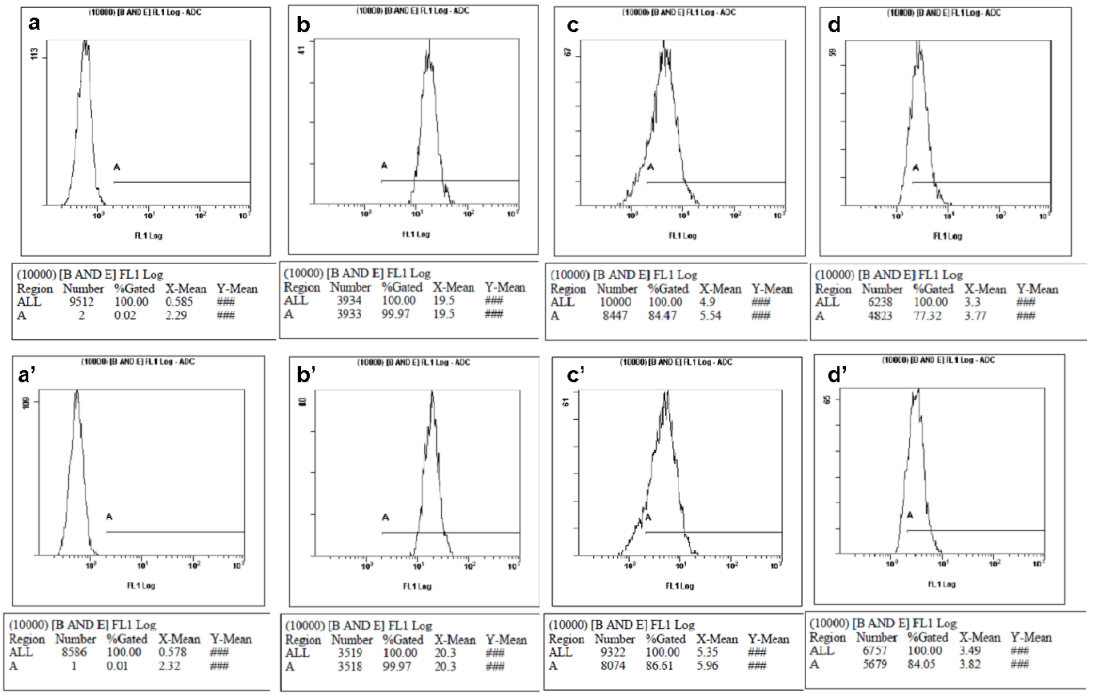

(10000) [B AND E] FL1 Log

Region	Number	%Gated	X-Mean	Y-Mean
ALL	9512	100.00	0.585	###
A	2	0.02	2.29	###

(10000) [B AND E] FL1 Log

Region	Number	%Gated	X-Mean	Y-Mean
ALL	3934	100.00	19.5	###
A	3933	99.97	19.5	###

(10000) [B AND E] FL1 Log

Region	Number	%Gated	X-Mean	Y-Mean
ALL	10000	100.00	4.9	###
A	8447	84.47	5.54	###

(10000) [B AND E] FL1 Log

Region	Number	%Gated	X-Mean	Y-Mean
ALL	6238	100.00	3.3	###
A	4823	77.32	3.77	###

(10000) [B AND E] FL1 Log

Region	Number	%Gated	X-Mean	Y-Mean
ALL	8586	100.00	0.578	###
A	1	0.01	2.32	###

(10000) [B AND E] FL1 Log

Region	Number	%Gated	X-Mean	Y-Mean
ALL	3519	100.00	20.3	###
A	3518	99.97	20.3	###

(10000) [B AND E] FL1 Log

Region	Number	%Gated	X-Mean	Y-Mean
ALL	9322	100.00	5.35	###
A	8074	86.61	5.96	###

(10000) [B AND E] FL1 Log

Region	Number	%Gated	X-Mean	Y-Mean
ALL	6757	100.00	3.49	###
A	5679	84.05	3.82	###

Figure S8. Flow cytometry. Before addition of PI: (a) medium, (b) **1a**, (c) **1b** and (d) **1c**. After addition of PI: (a') medium, (b') **1a**, (c') **1b** and (d') **1c**. All samples were measured after addition of 1N HCl to reach pH 6.

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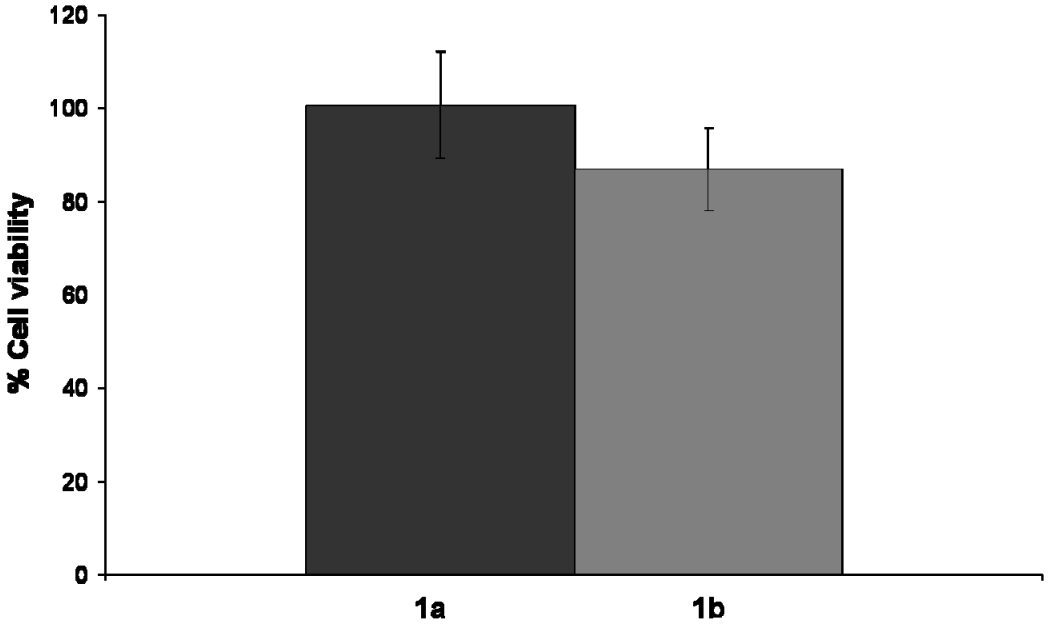

Figure S9. Viability of HeLa cells in the presence of the SPION-peptide conjugates **1a** and **1b** at 1 μM of CF measured by MTT assay. Cells were incubated for 6 h at 37 ºC under 5% CO_2 atmosphere and MTT (0.5 mg/mL) was added 2 h before the end of incubation.

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