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**SUPPORTING INFORMATION**

# Polymer coated inorganic nanoparticles: tailoring the nanocrystal surface for designing nanoprobes with biological implications

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## Experimental section

### *Materials*

Poly(maleic anhydride-*alt*-1-octadecene) MW 30000-50000, poly(isobutylene-*alt*-maleic anhydride), poly(styrene-co-maleic anhydride) cumene terminated, dodecylamine, oleylamine, bis(6-aminohexyl)amine, *N,N'*-dimethylethylendiamine, *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), *O,O'*-Bis(2-aminoethyl)octadecaethylene glycol (diamine PEG), fluorescein isothiocyanate, Fluorescent red Mega 480 NHS ester, folic acid, the human FITC-IgG and the anti-Annexin V IgG were purchased from Sigma. Poly(maleic anhydride *alt*-1-octadecene) MW 20000-25000 was purchased from Polysciences. TAT-cysteine peptide was purchased from Chinese Peptide.

### *Synthesis and polymer coating procedure*

Iron oxide nanoparticles (8, 11 and 12 nm sized) were synthesized according to a previously reported procedure with minor changes. [1]

Seven polymer molecules were employed to transfer the IONPs into water by the polymer coating method. The structure and the molecular weights of these polymers are reported in Table 1S.

| polymer | structure | MW (g/mole) | Monomer units/ nanoparticle nm$^2$ |
|---|---|---|---|
| P1 | Poly(maleic anhydride-alt-1-octadecene) | 30000-50000 | 500 |
| P2 | Poly(maleic anhydride-alt-1-octadecene) | 20000-25000 | 400 |
| P3 | Poly(isobutylene-alt-maleic anhydride) grafted with dodecylamine at a percentage of reacted anhydrides equal to 50% | 9700 (assumed) | 100 |
| P4 | Poly(isobutylene-alt-maleic anhydride) grafted with dodecylamine at a percentage of reacted anhydrides equal to 75% | 11300 (assumed) | 100 |
| P5 | Poly(isobutylene-alt-maleic anhydride) grafted with oleylamine at a percentage of reacted anhydrides equal to 50% | 11300 (assumed) | 100 |
| P6 | Poly(isobutylene-alt-maleic anhydride) grafted with oleylamine at a percentage of reacted anhydrides equal to 75% | 13700 (assumed) | 100 |
| P7 | Poly(styrene-co-maleic anhydride), cumene terminated | 1900 | 100 |

**Table 1S** Structure and molecular weight of the maleic anhydride polymers and copolymers employed in this study. The fourth column reports the amount of monomer units used per nanoparticle nm$^2$ in the polymer coating procedure.

P1,P2 and P7 were used as received, while P3 and P4 were prepared grafting dodecylamine molecules to the poly(isobutylene-*alt*-maleic anhydride) polymer molecules according to a previously reported procedure. [2].

Two grafting ratios were used: either 50% or 75% of the anhydride groups of the polymer were reacted with the alkylamine. Oleylamine was used as alkylamine for the preparation of P5 and P6. The molecular weights of P3, P4, P5 and P6 reported in table 1S are based on the assumption that all the alkylamine molecules added to the polymer solution covalently attached to the polymer backbone.

The polymer coating method here employed has been already described. [3] The conditions and the parameters of the coating procedure have been kept constant for all the polymer molecules under investigation, except for the ratio of monomer units added per nanoparticle $nm^2$ (see column 4 of table 1S).

Briefly, a solution of polymer in chloroform was added to a solution of oleic acid coated IONPS in chloroform, at a concentration of 0.2 μM, up to reach a ratio of polymer monomer units per $nm^2$ of nanocrystal surface corresponding to that reported in the table. After evaporation of the solvent under reduced pressure, a solution of bis(hexamethylene)triamine in chloroform was added to the dried sample in order to reach a ratio of cross-linker molecules per $nm^2$ of nanocrystal equal to 10. After mild sonication for 30 minutes, the solvent was removed under reduced pressure. Then sodium borate buffer (50 mM, pH 9) was added to the dry film and the resulting mixture was sonicated at 60°C for 2 hours, thus obtaining the nanoparticles dissolved in aqueous solution. In order to remove the unbound polymer, the nanoparticle solution was concentrated to a few millilitres and was then ultra-centrifuged at 150000 rcf on a continuous sucrose gradient (10-60%) for 200 minutes. Finally the layer of nanoparticles was recovered from the gradient and washed three times with the buffer through centrifugal filters in order to remove the sucrose.

***Stability tests***

IONPs were diluted in water having either different pH values (from 4 to 10) or different ionic strength (from 1 to 400 mM NaCl) in order to reach a final concentration equal to 2 μM. The solutions were left under stirring for 3 days and thereafter the stability of the samples was visually verified.

***Functionalization of the polymer shell***

**(i) Electrostatic binding**

Preparation of thionin-IONPs. 1 mL of polymer coated IONPs at a concentration of 1 μM were added to 500 μL of an aqueous solution containing 1 mM thionin acetate. The solution was left under stirring over night. Thereafter, in order to remove the excess unbound fluorophore, the solution was purified through prepacked Sephadex columns two times and concentrated on centrifugal tubes.

**(ii) Covalent binding**

Preparation of negatively and positive charged nanoparticles. In order to tune the surface charge of the IONPs, different amounts of *N*,*N*-dimethylethylendiamine molecules were bound to the carboxy groups of the polymer shell by varying the molar ratio of tertiary amine and EDC added per nanoparticle. For simplicity, only one reaction will be described in detail: 50 μL of a nanoparticles solution in borate buffer (2 μM) was mixed with 50 μL of *N*,*N*-dimethylethylendiamine solution in borate buffer (molar ratio of

amine/NP equal to 500). Then, 50 µL of EDC solution (molar ratio of EDC/NP equal to 12500) was added. The solution was allowed to react for 3 h at room temperature under vigorous stirring and the unreacted *N*,*N*-dimethylethylendiamine molecules were removed by washing the sample several times on centrifuge tubes (30000 MWCO). For the preparation of the other samples the molar ratios were adjusted as follows: amine/NP molar ratios equal to 2500, 5000, 10000, and EDC/NP molar ratios equal to 25000, 50000, 100000, 200000, 250000, 400000, 500000, 600000, 800000 and 100000 as showed in figure 10. In the control sample, EDC was not added to the reaction mixture.

Preparation of FA-IONPs. 500 µL of a solution containing diamine PEG-IONPs (5 µM) were mixed with an equal volume of folic acid (1 mM in borate buffer) and EDC (150 mM in borate buffer). The reaction was kept under stirring for 5 hours at 4 °C. Soon after the FA-IONPs were purified from the unbound folic acid by means of size exclusion chromatography (Sephadex columns) and by washing the sample on centrifuge filters three times.

Preparation of TAT-IONPs. The TAT peptide used in this study has been modified by the addition of cysteine residue. The complete amino acid sequence is the following: Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Gly-Cys. Sulfo-SMCC was used as cross-linking molecule to bind the thiol group of the cysteine residue to the diamine PEG-IONPs according to the following protocol: a solution containing diamine PEG-IONPs (500 µL 5 µM) was mixed with the sulfo-SMCC (500 µL 2.5 mM) for 40 minutes at 4 °C. Soon after the unbound crosslinker was removed by means of a purification step through a Sephadex column. The collected sample was concentrated to 1 mL through centrifuge filters and the TAT peptide (500 µL 1 mM) was added to the solution. The reaction mixture was kept under stirring O.N. at 4 °C. Finally the TAT-IONPs were purified from the unbound peptide by means of size exclusion chromatography and by washing the sample on centrifuge filters three times.
Similar reaction conditions were set for the preparation of CdSe/CdS QRs functionalized with either FA or TAT peptide. The QRs were prepared according to a reported method [4].

Preparation of IgG -NPs. The human IgG-FITC (100 µL 5.5 mg/mL) or the anti annexin V IgG (100 µL 2.3 mg/mL) were added to 350 µL of a solution containing diamine PEG-NPs at a concentration equal to 2 or 1 µM, respectively. Then 350 µL of a solution of 100 mM EDC dissolved in borate buffer were added and the reaction mixture was left under stirring at 4 °C O.N.. Thereafter the IgG-FITC conjugated nanoparticles were passed through a Sephadex column three times in order to remove the unbound protein.

***Dynamic Light Scattering and Zeta Potential measurements***

DLS and Zeta potential measurements were performed on a Zetasizer Nano ZS90 (Malvern, USA) equipped with a 4.0 mW He−Ne laser operating at 633 nm and with an avalanche photodiode detector. Measurements were made at 25 °C in water, adjusting the pH to 7 with a NaOH solution 0.1 M.

***Gel Electrophoresis characterization***

Electrophoretic characterization was carried out by running the nanoparticles through a 2% agarose gel immersed in TBE buffer (pH 8.0) for a run time of 1 h at 100 V, after which the gel was observed under visible light. Before the loading step on the gel, to each sample a solution of Orange G and 30% glycerol in a gel-loading buffer (corresponding to 20% of the sample volume) was added.

***Optical characterization***

UV-vis absorption and Photoluminescence spectra were recorded using a Varian Cary 300 UV-vis spectrophotometer and a Cary Eclipse spectrofluorimeter.

***Cell lineage maintainance and viability assay***

HeLa (ATCC-CCL-2) and KB cell lines (ATCC-CCL-17) were grown at 37 °C under 5% $CO_2$ atmosphere in, respectively, DMEM and RPMI-1640 media, supplemented with L-glutamine (2 mM), penicillin (100 units $mL^{-1}$), streptomycin (100 mg $mL^{-1}$), and 10% heat-inactivated fetal bovine serum (FBS).

For the viability assay (MTT test) 1.5 x $10^5$ Kb cells or 5 x $10^4$ HeLa cells suspended in 0.5 mL of serum supplemented medium were seeded in a 24 multiwell plate and after 24 hours the medium was replaced with 0.5 mL of positively or negatively charged IONP containing medium at three different concentration of iron (10, 25 and 50 μg/mL). Cells were incubated at 37°C for 6 hours, then the medium was removed, the cells were washed three times with PBS and analyzed directly or after additional 18 and 42 hours of incubation with fresh medium. Then, 0.5 mL of fresh serum-free medium containing 1 mg/mL of Thiazolyl Blue Tetrazolium Bromide 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 0.5 mL of DMSO, leading to a violet solution whose absorbance at 570 nm was determined. Each assay was repeated 4 times and the values were compared to the absorbance value of the untreated control cells.

***Determination of the Intracellular Fe Concentration***

Cells (2 x $10^5$ HeLa cells and 3 x $10^5$ KB cells) incubated with the negatively charged IONPs (Zeta potential at pH 7 = -31.1 mV) and positively charged IONPs (Zeta potential at pH 7 = +16.6 mV) at different extracellular Fe concentrations (10, 25, 50 μg/mL of Fe) were seeded in each well of a 6 well-plate in 2 mL of culture medium. After 24 hours the medium was replaced with 1 mL of fresh medium containing the iron oxide samples and the cells were incubated at 37 °C for 6 hours. Then the cells were washed three times with PBS and trypsinized. The cell suspension was then centrifuged, the supernatant removed and 2 mL of a concentrated HCl solution was added to digest the cells. The intracellular Fe concentration was measured by means of elemental analysis (ICP-OES spectrometer, iCAP 6500, Thermo).

In a distinct experiment 1.5 x $10^5$ KB cells incubated with 30 nM FA-IONPs and TAT-IONPs (corresponding to 17 μg/mL of Fe) were seeded in each well of a 6 well-plate in 2 mL of culture medium.

After 24 hours the medium was replaced with 2 mL of fresh medium containing the iron oxide samples and the cells were incubated at 37 °C for 1 hour or 24 hours. Then we proceeded as described above.

***Electron microscopy characterization***

Low-Magnification TEM images were recorded on a JEOL Jem1011 microscope operating at an accelerating voltage of 100 KV.

The nanocrystals samples were prepared by dropping a dilute solution of particles on carbon coated copper grids.

**Preparation of cellular samples for TEM characterization**

4 x $10^5$ cells suspended in 8 mL of medium were seeded in a culture dish. After 24 h incubation at 37 °C the medium was replaced with 8 mL of fresh medium containing either the FA-IONPs or the TAT-IONPs at a concentration equal to 10 nM (corresponding to 5.7 µg/mL of Fe). Cells were then incubated at 37 °C for either 1 hour or 24 hours. Then, they were washed with PBS and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 4°C for 30 min. The fixed specimens were washed three times with the same buffer and 1% osmium tetroxide in cacodylate buffer was added for 1hour. Thereafter, the cells were washed again and dehydrated with 25%, 50%, 75% and 100% acetone. Two steps of infiltration in a mixture of resin/acetone (1/1 and 2/1 ratios) followed and finally the specimens were embedded in 100% resin at 60 °C for 48 h. Ultrathin sections (80 nm thick) were cut on an Ultramicrotome (Leika), stained with lead citrate and observed under the electron microscope.

***Confocal imaging***

2 x $10^5$ cells suspended in 4 mL of medium were seeded in a culture dish. After 24 hours incubation at 37 °C the medium was replaced with 4 mL of fresh medium containing either the FA-QRs or the TAT-QRs at a concentration equal to 20 nM. After either 1 hour or 24 hours incubation, cells were first washed with PBS, fixed with 4% paraformaldeyde and then imaged under the confocal microscope, an Olympus FV-1000 microscope equipped with an argon laser source (excitation at 488 nm) with a DM488/405-type dichroic filter. An acquisition window at 600 ± 20 nm was set.

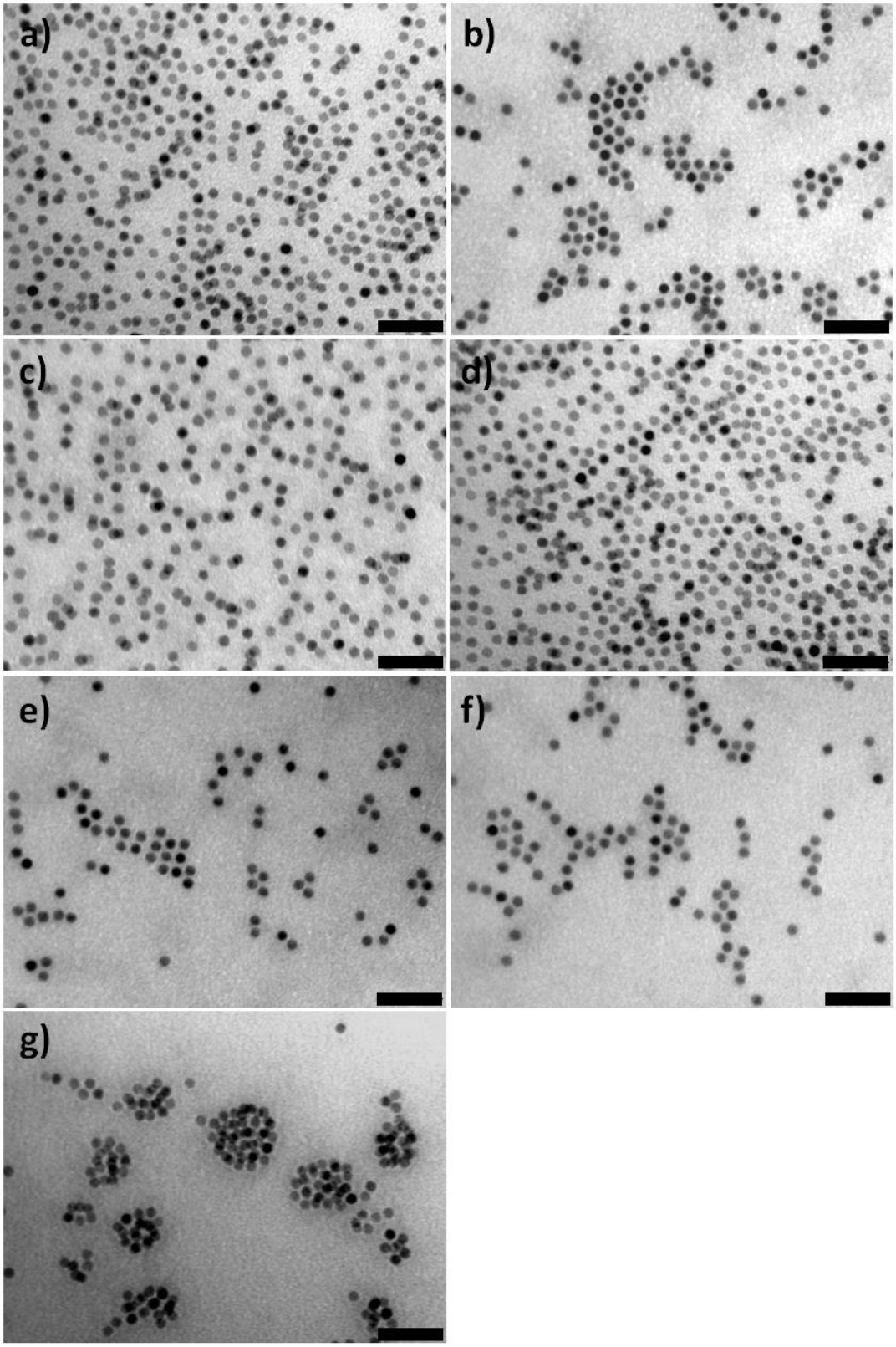


**Figure 1S**: TEM characterization of 11 nm sized iron oxide nanoparticles transferred into water by coating them with seven different polymer: P1 (a), P2 (b), P3 (c), P4 (d), P5 (e), P6 (f), P7 (g). Scale bar is 50 nm.

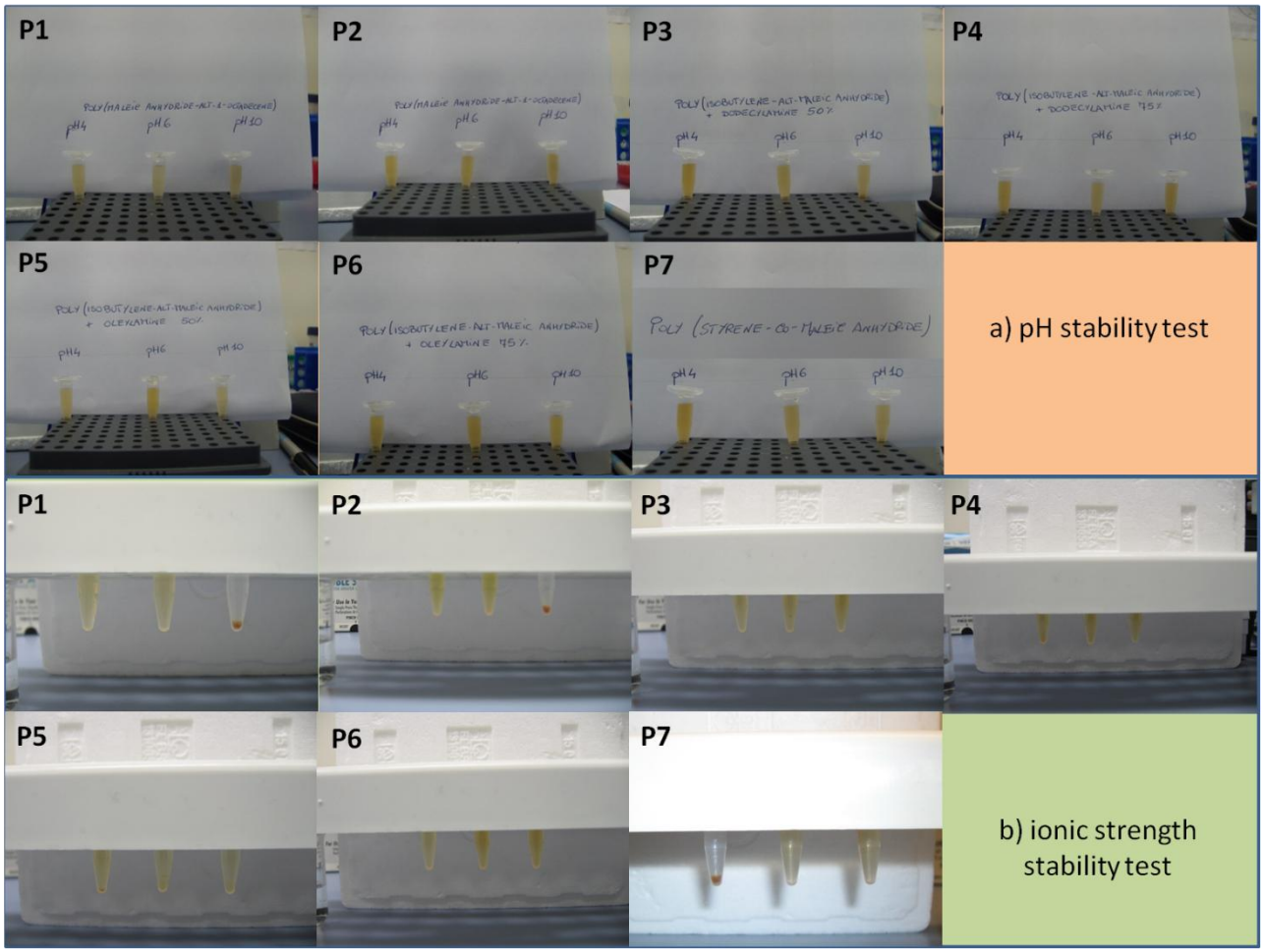


**Figure 2S**: a) pH and b) ionic strength stability tests. Three pH points (4, 6 and 10) and three ionic strength values (1, 50 and 400 mM NaCl) were assayed per each type of sample.

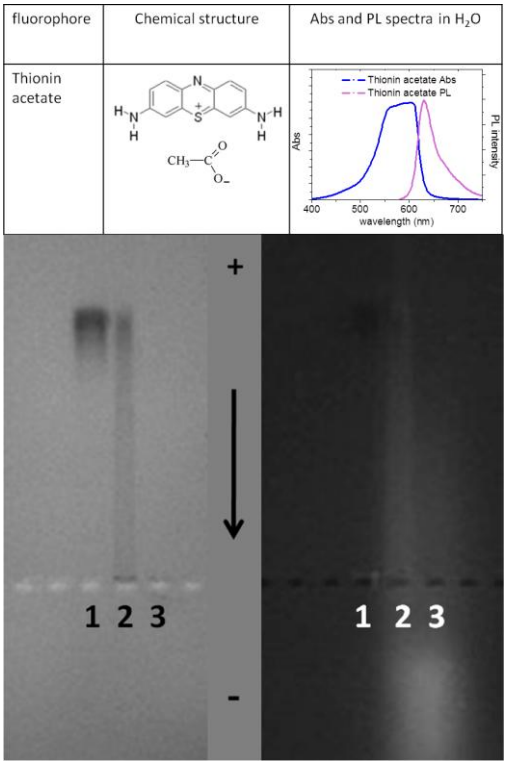

**Figure 3S**: in the upper part of the image the structure and the Abs/PL spectra of thionin acetate are shown, while in the lower part the gel electrophoresis characterization of polymer coated iron oxide nanoparticles electrostatically functionalized with thionin acetate is reported. Left panel corresponds to the gel image under bright filed, while the right panel to the image acquired under UV excitation with a 620±20 filter. Lane 1 corresponds to the migration of the polymer coated nanoparticles, while lane 2 to the thionin functionalized nanoparticles. Lane 3 shows the migration of the free thionin.

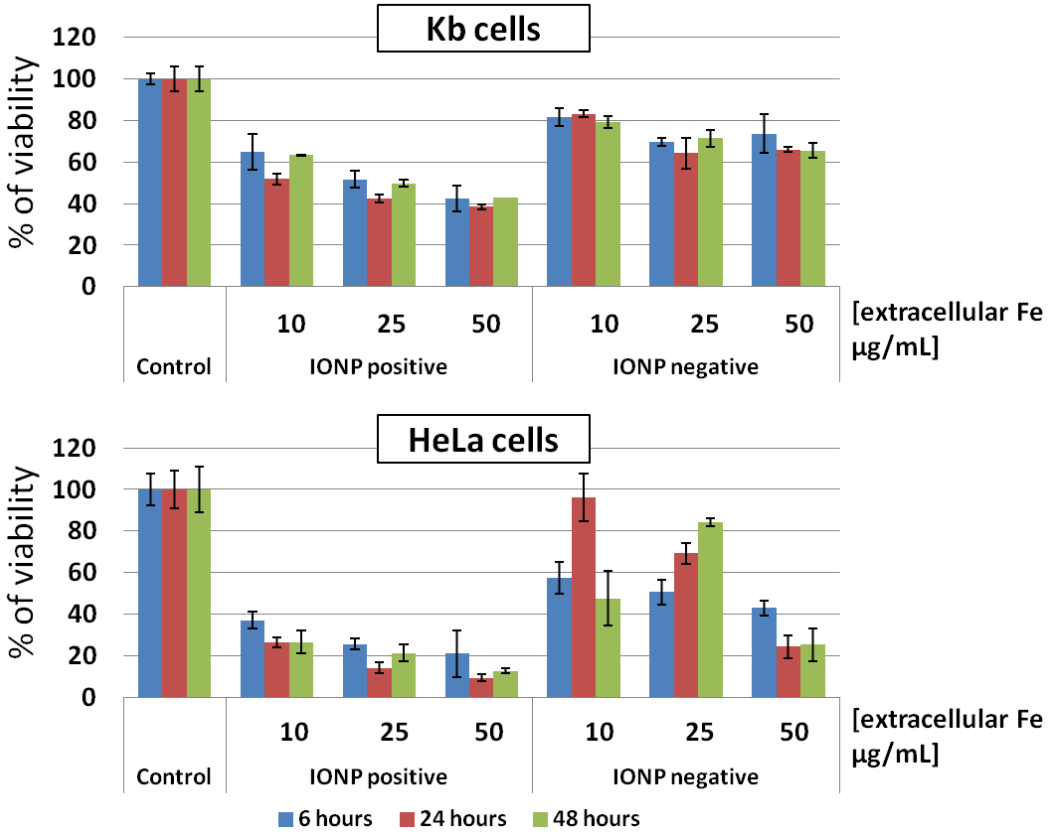


**Figure 4S**: Viability assay performed using two tumor cell lineages, KB cells and HeLa cells. The cells were incubated with positively and negatively charged IONPs for 6, 24 and 48 hours.

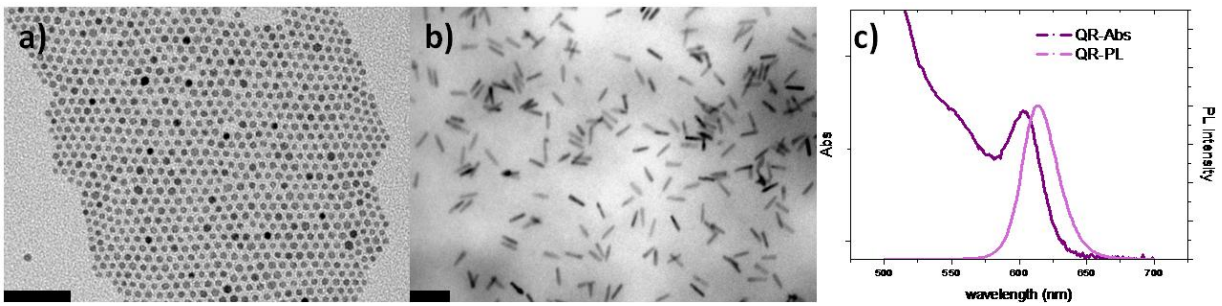


**Figure 5S**: TEM images of the iron oxide NPs a) and of the CdSe/CdS QRs b) functionalized with the TAT-cysteine peptide. c) shows the absorption and photoluminescence spectra of the QRs employed in this work. Scale bar is 50 nm.

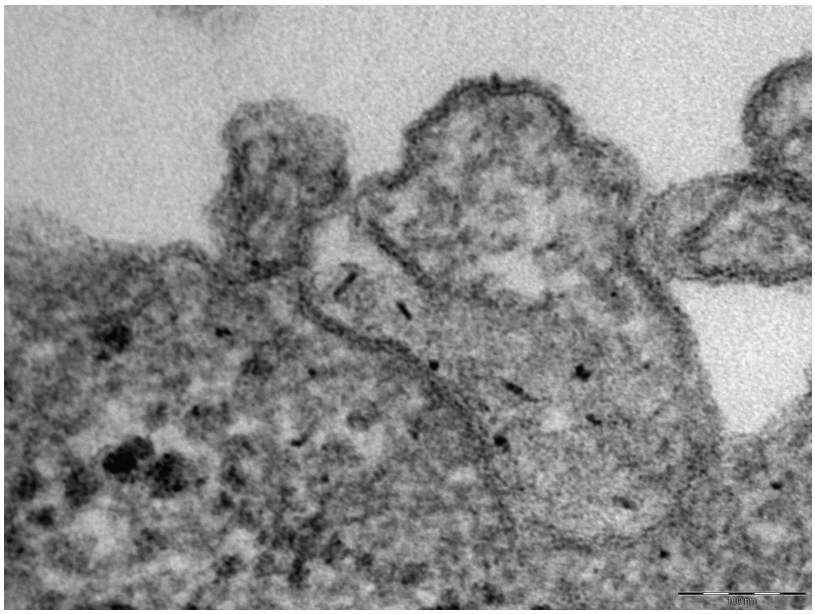

**Figure 6S**: TEM image of a KB cell incubated with the QR-FA conjugate for 1 hour.

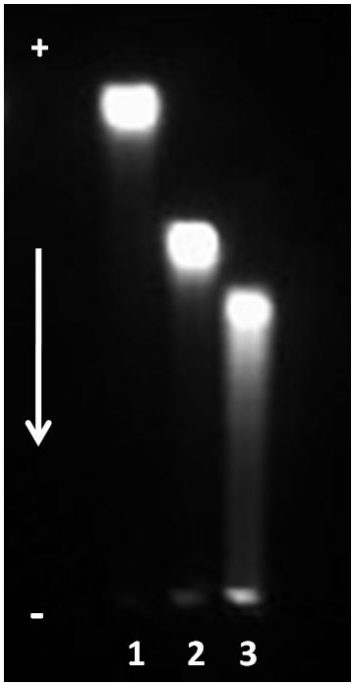


**Figure 7S**: Gel electrophoresis characterization of the anti annexin V IgG-modified QRs. Lane 1 corresponds to the starting polymer coated QRs, lane 2 to the PEG functionalized sample, while lane 3 shows the mobility of the QRs conjugated to the human antibody.

## References

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4. Carbone, L. et al. Synthesis and micrometer-scale assembly of colloidal CdSe/CdS nanorods prepared by a seeded growth approach. *Nano Lett.* **7**, 2942-2950 (2007).