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Supporting Information

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dsRNA Functionalized Multifunctional g-Fe_2O_3 Nanocrystals : A Tool for Targeting Cell Surface Receptors

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Characterization of the maghemite nanoparticles

Phase identification of the naked iron oxide nanoparticles was carried out using transmission electron microscopy (TEM) (Philips 420 instrument with an acceleration voltage of 120 kV), magnetic susceptibility (SQUID) measurements and Mössbauer spectroscopy (Figure1). The TEM image of the unfunctionalized γ-Fe_2O_3 nanoparticles in Figure 1a shows well separated spherical γ-Fe_2O_3 nanocrystals with a narrow size distribution ranging from 7 to 10 nm. Figure 1b shows the high resolution TEM (HRTEM) image (Philips TECNAI F30 electron microscope, field-emission gun, 300 kV extraction voltage) of a single nanocrystal with interlayer *d*-spacings of 0.25 nm which can be attributed to the (311) plane of maghemite.

Maghemite (γ-Fe_2O_3) and magnetite (Fe_3O_4) cannot easily be distinguished by Mössbauer spectroscopy as both of them adopt an inverse spinel structure. The Mössbauer spectrum of the unfunctionalized nanoparticles at room temperature is shown in Figure 1c. As maghemite is superparamagnetic for particles sizes < 9 nm the Mössbauer spectrum contains only a single doublet. The Mössbauer parameters at room temperature (IS = 0.37(3) mm s^{-1}, QS = -0.35(2) mms^{-1}) are compatible with bulk ferrimagnetic maghemite.[19] The quadrupole splitting deviates slightly from the literature value because of the large surface fraction of nanoparticles compared to their volume. The hysteresis loop for the γ-Fe_2O_3 nanocrystallites in Figure 1d indicates superparamagnetic behavior without any hysteresis at room

temperature. The magnetization data indicate a magnetization of 65 emu/g for the γ-Fe_2O_3 nanoparticles at 40 kOe.

[19] (a) N. N. Greenwood, T. G. Gibb, Mössbauer Spectroscopy, Chapman and Hall Ltd, London, 1971. (b) R. M. Taylor, U. Schwertmann, *Clay Minerals* **1974**, *10*, 299-310.

Characterization of the functionalized maghemite nanoparticles

Figure S1 shows overview images of naked and polymer functionalized nanoparticles. The functionalization of the maghemite nanoparticles with multifunctional polymeric ligand was characterized by UV-VIS spectroscopy as shown in Figure S3. The absorption spectrum of the composite contains the respective absorption of the polymeric ligand. Figure S2a shows the polymer functionalized maghemite nanoparticles carrying a NBD dye under normal light, Figure S2b shows the corresponding fluorescence image.

Experimental Section

Synthesis of γ-Fe_2O_3 nanoparticles. Iron pentacarbonyl, $Fe(CO)_5$ (0.2 ml 1.52 mmol) was injected into a solution containing 0.91 g of lauric acid (4.56 mmol), 7 ml of octyl ether, and 0.57 g of dehydrated trimethyl nitrooxide $(CH_3)_3NO$ (7.60 mmol) at 100 °C in an argon atmosphere, with vigorous stirring. As soon as $Fe(CO)_5$ was injected into the mixture, the temperature increased to 120 °C and the solution became dark-red, which indicated the successful oxidation of $Fe(CO)_5$. The reaction mixture was then stirred for 1 h at 120 °C, and the solution was slowly heated to reflux. The solution colour gradually became black, indicating that nanoparticles were being formed. After refluxing for 1 h, the solution was cooled to room temperature, and a black precipitate was obtained upon adding excess ethanol and centrifuging. The precipitate could easily be redispersed in octane or toluene. [12] The sample was analyzed by magnetic susceptibility measurements (SQUID), (high

resolution) electron microscopy (HRTEM), Mössbauer (Figure 1) and UV/Vis spectroscopy (Figure S3)

Synthesis of the multifunctional copolymer. For the synthesis of the multifunctional poly (acrylamides), poly (active ester) PFA (300 mg, 1.26 mM repeating units) was dissolved in 10 ml dry DMF. Piperazinyl-4-chloro-7-nitrobenzofurazane (pipNBD) (8 mg) dissolved in dry DMF was added, and the resulting solution was stirred for 2 h at 50°C. A solution of 3-hydroxytyramine hydrochloride (24 mg) in 1.5 ml DMF and 0.1 ml triethylamine was added and the clear mixture was stirred for 3 h at 50°C. After that a solution of 1-Boc-1,4-diaminobutane (240 mg) in DMF (4 mL) was added and the solution again stirred for 5 h at 50°C. The solution was concentrated to about 5 mL in vacuum and polymeric ligand was precipitated by cold ethyl ether. The precipitated polymer was centrifuged and the solvent was decanted. After drying, 286 mg of a dark brown solid was obtained. [10b]

Cleavage of the Boc group. The polymer was dissolved in CH_2Cl_2 (30 mL). After that trifluoroacetic acid (2 ml) was added. The mixture was stirred at room temperature for 2 h. After that 2 N HCl (40 ml) was added and the phases separated. The organic phase was extracted with 2 N HCl (2 x 20 mL). The combined aqueous phases were evaporated under vacuum and dialysed against water until the pH of the outer solution was 7. Finally, product was dried under vacuum. The reaction yielded 200 mg of the polymer.

Polymer functionalization of the γ-Fe_2O_3 nanoparticles. 15 mg of the above synthesized γ-Fe_2O_3 nanocrystallites were treated with 50 mg of the reactive polymer dissolved in N,N-dimethylformamide (DMF). The reaction was carried under inert atmosphere and vigorous mechanical stirring at 50 °C for 12h followed by cooling the reaction system to room temperature. To remove unbound polymer the coated magnetic particles in the solution were extracted by a magnetic particle concentrator (Dynal MPC1-50, Dynal Biotech, France) at room temperature. The isolated magnetic nanoparticles were washed with DMF ensuring the removal of unreacted polymer and fluorescent analysis performed using an Olympus AHBT3 light microscope, together with an AH3-RFC reflected light fluorescence attachment at the emission wavelength of 530 nm.

Coupling of poly (IC) onto polymer functionalized g-Fe_2O_3 nanoparticles. dsRNA, poly(IC), was obtained commercially and prepared to a final concentration of 2 mg/ml. The reaction mechanism between primary amine and a phosphate group using 1-ethyl-3-[3-dimethylamino propyl]carbodiimide (EDC) in the presence of imidazole buffer (MeIm, 0.1 M, pH 7.5) as described elsewere.[11,13] In a typical experiment 38 µl of EDC (0.013 M stock solution in MeIm buffer) was mixed with 10 µl of (poly I:C) (1:10 dilution in MeIm, 0.1 mM, pH 7.5 buffer). The activated dsRNA mixture was coupled to 20 µl of polymer functionalized maghemite carrying amine moieties. As a control, polymer functionalized γ–Fe_2O_3 carrying dsRNA was heated up to 75ºC for 5 minutes. The aliquots were immediately freezed. All the above mentioned experiments were carried out in RNAse free solutions and environment. 10 µl of each sample were loaded into a 1% agarose gel (TBE buffer) and the run was carried out at 60 V for 1 h. The gel was stained using ethidium bromide (2 µg/mL).

Caki-1 cell culture. Caki-1 cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ; Braunschweig, Germany). Caki-1 cells were grown in McCoy's 5A medium (Biochrom AG; Berlin, Germany), and supplemented with 10% Fetal Bovine Serum (Biochrom, AG Berlin, Germany), 1.14% penicillin/streptomycin (Biochrom, AG Berlin, Germany) and 1.14% non-essential amino acid solution (Biochrom AG, Berlin, Germany). Caki-1 McCoy's 5A medium was additionally supplemented with 0.7% L-glutamine (Biochrom AG, Berlin, Germany). Cell cultures were routinely grown in 75-cm^2 cell culture flasks and were maintained at 37°C, 5% CO_2 and 95% relative humidity. For subsequent immunostaining and nanoparticle incubations cells were grown on 6-well plates containing cover slips.

Isolation and culture of Human monocytes. Human peripheral blood mononuclear cells were isolated from healthy volunteers. Human monocytes were obtained by density gradient centrifugation with Histopaque ®-1077 (Art. No. H8889, Sigma, Germany) as described by the manufacturer. Briefly, 5 mL of EDTA(2.7%) containing solution was layered on the top of the 5 mL of Histopaque® -1077 and the 2 layers were centrifuged at 400x*g* for 30 minutes at room temperature. Mononuclear cells were collected at the interface and washed trice with

isotonic phosphate buffered saline solution and centrifuged at 250x*g* for 10 minutes at room temperature. Cells were cultivated in RPMI-1640 medium (Biochrom AG, Berlin, Germany) supplemented with 10% Foetal Bovine Serum (FBS) (Cat. No. A11-041, PAA Laboratories GmbH, Austria). Monocytes were purified by adherence to plastic tissue 6 multiwell culture plates (Art. No.353046, Falcon, Becton Dickinson; USA) for 2hrs and maintained at 37°C, 5% CO_2 and 95% relative humidity.

RT-PCR of TLR3 in Caki-1. The RNA was isolated from the Caki-1 cells using the RNA STAT-60™ (Tel-Test Inc.; Friendswood, TX, USA), according to the company's protocol for RNA isolation. The RNA was purified using a DNA-free™ purification kit (Ambion Ltd.; Cambridgeshire, UK). The integrity of the isolated RNA was checked by standard gel electrophoresis with 1% agarose in Tris-Acetate-EDTA buffer (pH 8.0). The total RNA was reverse transcribed into cDNA using a SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen™ Ltd.; Paisley, UK), according to the manufacturer's guidance. cDNA was then amplified by PCR. The sense and antisense primer sequences used for the TLR3 detection were 5'- AACTGGATATCTTTGCCAATTCAT and 3'-TGGCTGTTACTCAGAGACAGATTC, respectively (Operon Biotechnologies GmbH; Cologne, Germany). The PCR was carried out using the Expand™ High Fidelity PCR System kit (Roche Diagnostics; Mannheim, Germany). Samples were initially heated for 4 min and 94°C and PCR amplification cycling conditions were the following: 1 min denaturation at 94°C, 2 min annealing at 60°C and 3 min extension at 72°C, for a total of 35 cycles. Subsequently, the amplified PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining (2 µg/mL) along with a 100 bp DNA ladder (GeneRuler™, Fermentas GmbH; Leon-Rot, Germany). As positive control, mRNA hTLR3 of Caco-2 cells was analyzed and the cells cultured as described.[16]

Immunostaining of Caki-1 cells. Caki-1 cells were cultured until reaching confluence, as described [14]. The cells were washed three times with PBS followed by the treatment with a blocking solution of 3% Bovine serum albumin in PBS for 1 hr at RT. Cells were then incubated at 4 °C overnight with TLR3 mouse monoclonal antibody (1:50 dilution) (Prod no. TLR3.7, sc-32232, Santa Cruz Biotechnology, Inc., USA) in PBS solution containing bovine albumin serum (BSA) (3%) raised against full length TLR3 of human origin. Samples were

then washed with PBS and incubated with secondary antibody goat anti-mouse IgG-Texas Red conjugated (1:100 dilution prepared in 3%BSA/PBS) (Prod no. sc-2781, Santa Cruz Biotechnology, Inc., USA) for 2 hrs in dark. Nuclei were visualized by staining with 4,6-diamino-2-phenylindole (DAPI) (Cat. No. D1306, Invitrogen GmbH - Molecular Probes, Karlsruhe, Germany) for 10 min in the dark. Controls were performed incubating pre-immune serum with the cells for 1hr at RT. The fluorescence analysis was performed with an Olympus AHBT3 light microscope, together with an AH3-RFC reflected light fluorescence attachment at the emission wavelength of 456 nm and 620 nm to visualize the DAPI staining and the secondary antibody, respectively.

XTT test. A cell proliferation assay was performed using *in vitro* Toxicology Assay Kit, XTT based (TOX-2, Sigma, Steinheim, Germany). Caki-1 cells were cultured in 96-wells plate according to the cell culture procedure described previously[14] at $3x10^4$ cells/well. The cells were incubated with given concentrations of polymer functionalized γ-Fe_2O_3 nanoparticles (10, 50 and 100 μg/mL) in triplicate for 12 h. Cells were washed once with PBS and incubated with 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) in culture medium for 2 h under the following conditions: 37°C, 5% CO_2 and 95% relative humidity. XTT formazan crystal formation was measured spectrophotometrically at 450 nm using a Multi-well plate reader along with a reference at 690 nm.[18] The statistical data was calculated using OriginPro v7.5 software (OriginLab, Northampton, USA)

Multifunctional nanoparticles and Caki-1 cells. Polymer functionalized γ-Fe_2O_3 nanoparticles with surface bound dsRNA (poly(I:C)) were incubated confluent Caki-1 cells, for 3 hr at 37°C, 5% CO_2 and 95% relative humidity. Afterwards, the Caki-1 cells were washed three times with PBS and the cover slip was mounted onto a cover slide. Control sample included cells which were incubated with polymer functionalized γ-Fe_2O_3 without poly(I:C) bounded under the same conditions as for the poly(I:C)-polymer-γ-Fe_2O_3 complex. As control, human monocytes lacking TLR3 receptors at the surface were incubated with the poly(I:C)-polymer-γ-Fe_2O_3 complex for 3 hr at 37°C, 5% CO_2 and 95% relative humidity. The cell nuclei was stained with 4,6-diamino-2-phenylindole (DAPI). The fluorescence analysis

was performed with an Olympus AHBT3 light microscope, together with an AH3-RFC reflected light fluorescence attachment at the emission wavelength of 456 nm and 530 nm to visualize the DAPI staining and the γ-Fe_2O_3 functionalized with the multifunctional polymer, respectively. The images were co-localized using the software Analysis (Olympus).

Physical characterization. The resulting products were characterized by X-ray powder diffraction in reflection geometry, (Siemens D8 powder diffractometer, Cu-Ka radiation) and high-resolution transmission electron microscopy (Philips TECNAI F30 electron microscope; field-emission gun, 300 kV extraction voltage) equipped with a high-angle annular dark-field detector (HAADF), a Gatan imaging filter (GIF), and an energy dispersive X-ray analysis (EDX) system. Magnetic susceptibility measurements were performed using a Quantum Design MPMS-XL SQUID magnetometer. ^{57}Fe Mössbauer spectra were measured with a constant acceleration type Mössbauer spectrometer operated in a multichannel scaling mode. A 10 mCi ^{57}Co/Rh source was employed which was maintained at ambient temperature. The isomer shifts reported here are relative to metallic iron. The Mössbauer spectra were analyzed with the computer program EFFINO[20] using Lorentzian line shapes. Microscopic characterization was performed with an inverted laser scanning microscope (Leica TCS SL, Leica Microsystems, Bensheim, Germany).

[20] H. Spiering, L. Deák and L. Bottyán, *Hyperfine Interactions* **2000**, *125*, 197-204.

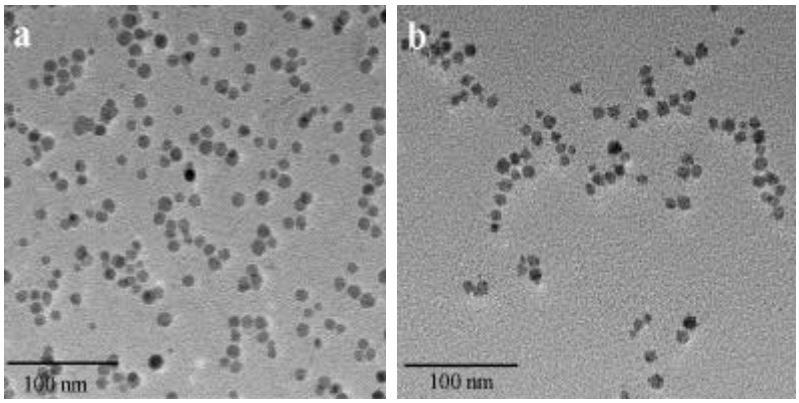


Figure S1. Transmission Electron Microscopy (TEM) images of naked (a) and functionalized (b) γ-Fe_2O_3 nanocrystals.

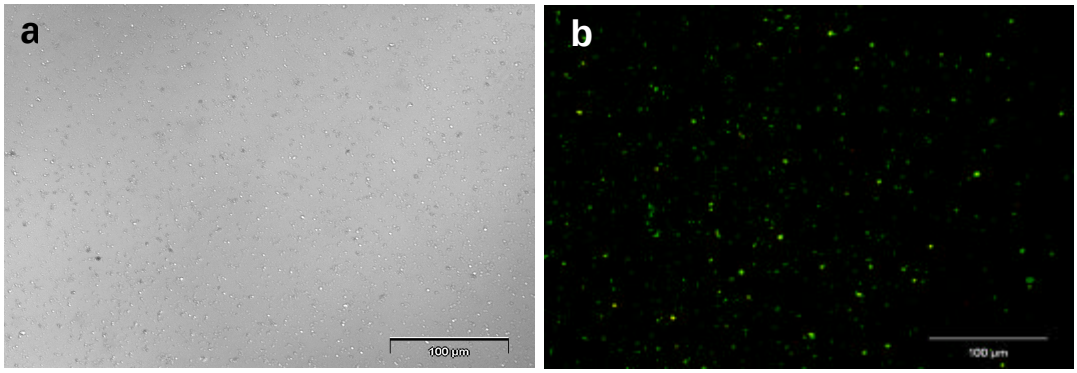


Figure S2. Light microscope images of (a) polymer functionalized γ-Fe_2O_3 nanocrystallites**,** (b) corresponding fluorescence image.

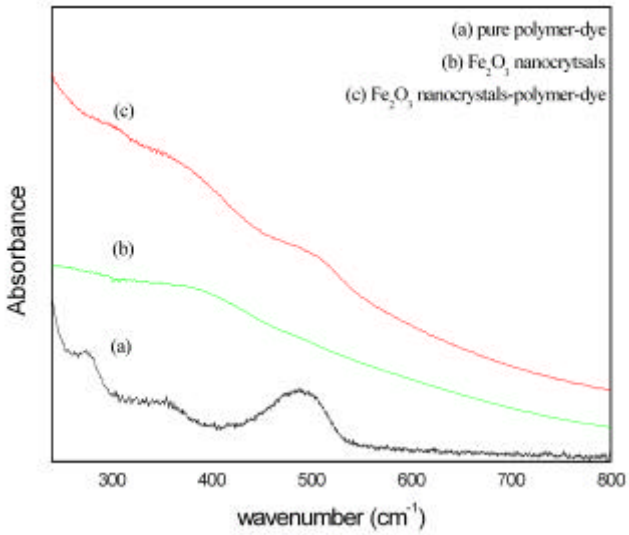


Figure S3. UV/Vis spectra of the polymer with (a) fluorescent NBD-dye, (b) unfunctionalized γ-Fe_2O_3 nanocrystals, and (c) γ-Fe_2O_3 nanocrystals with surface bound polymer/dye.

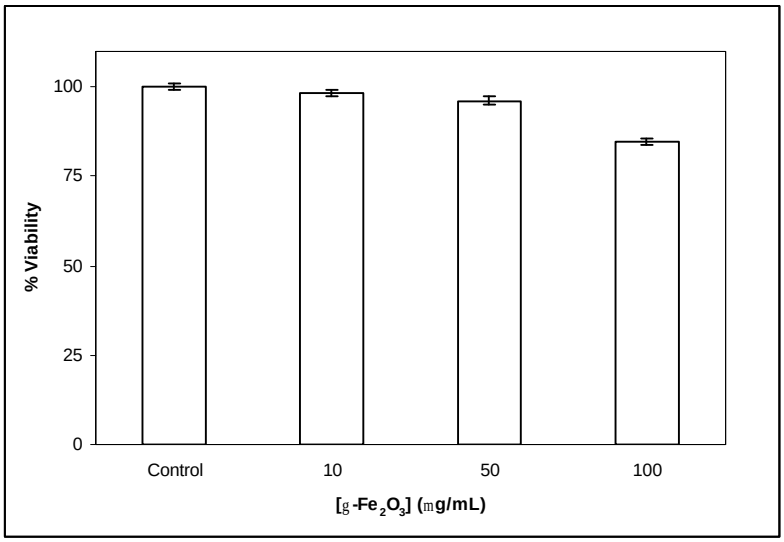


Figure S4. XTT cytoxicity assay of the percentage survival of cells exposed to various concentrations of polymer functionalized ?-Fe_2O_3 nanocrystals in comparison to that of the non-exposed control cells.

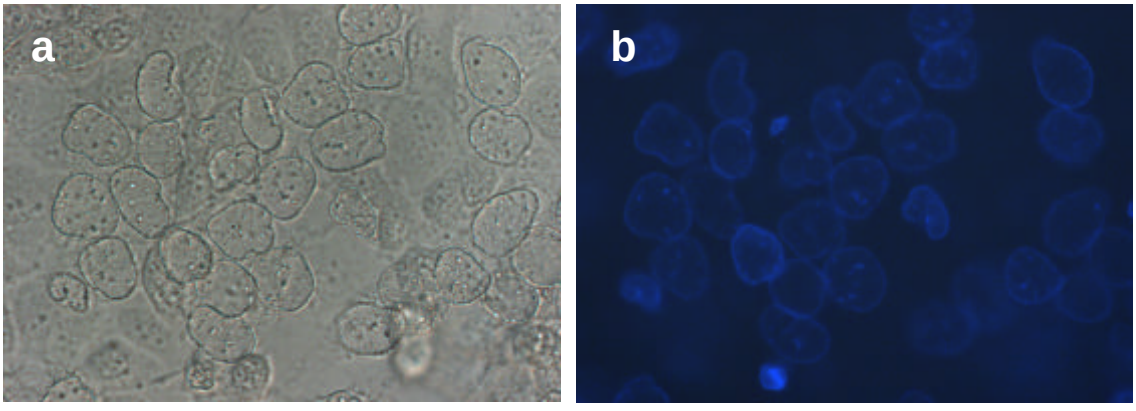
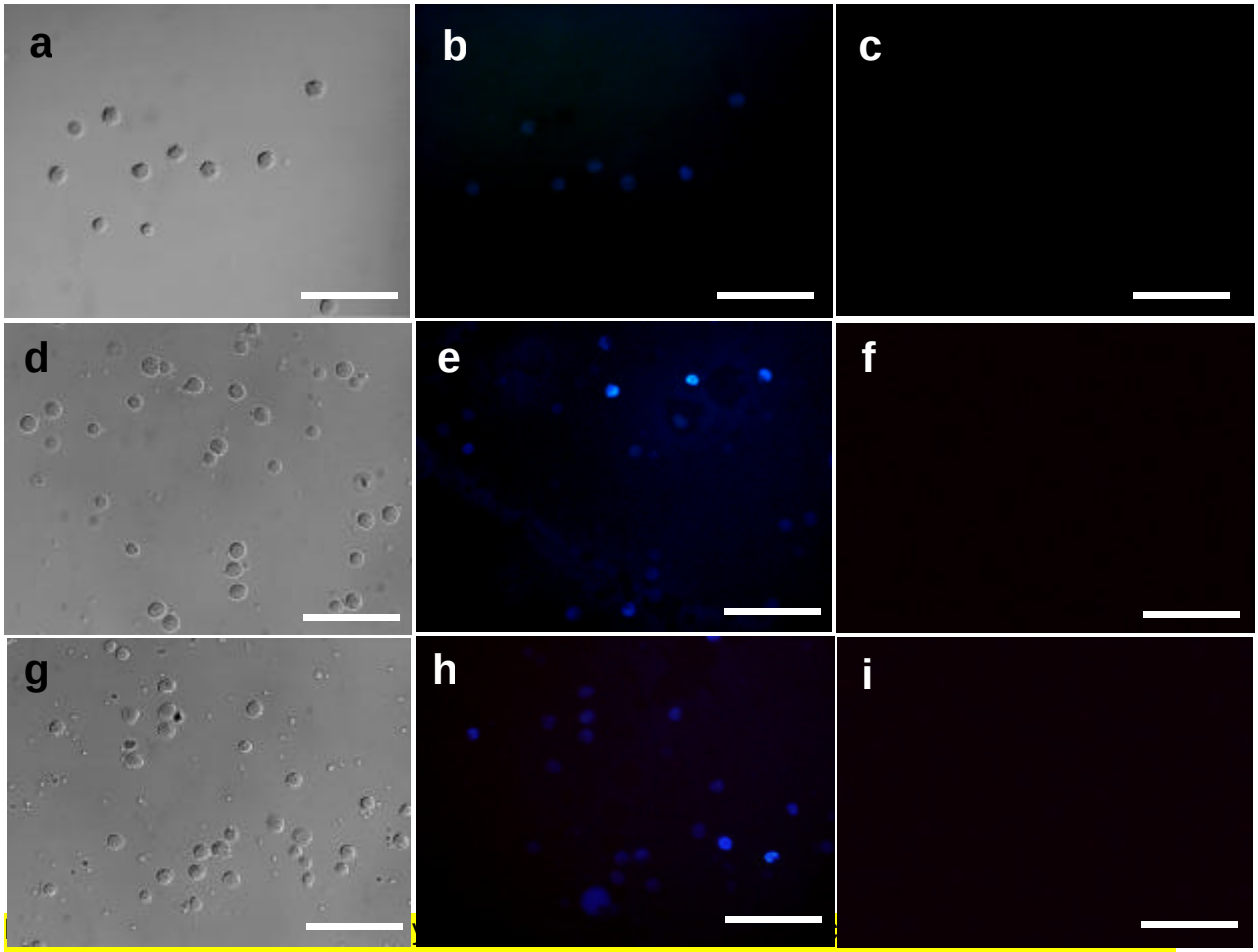


Figure S5. **(a)** Light microscopy images of Caki-1 cells incubated with multifunctional polymer functionalized γ-Fe_2O_3 nanocrystals in the absence of poly(I:C). **(b)** Corresponding fluorescent image, nuclei were visualized by staining with DAPI (blue).



surface (**d**) incubated with multifunctional polymer functionalized γ-Fe_2O_3 nanocrystals and (**g**) with poly(I:C). In the corresponding fluorescent images, **(b, e and h)** nuclei were visualized by staining with DAPI at the emission wavelength of 456 nm (blue). The corresponding controls (**c** (cells only)**, f and i**) performed at the emission wavelength of 530 nm (NBD-tagged polymer) remain dark as no particles with polymers carrying NBD are attached to the cells (scale bar: 50 μm).