Supporting Information

Folic acid on iron oxide nanoparticles: platform with high potential for simultaneous targeting, MRI detection and hyperthermia treatment of lymph node metastases of prostate cancer

Debora Bonvin,[a] Jessica A.M. Bastiaansen,[b,c] Matthias Stuber,[b,c] Heinrich Hofmann,[a] and Marijana Mionić Ebersold[a,b,c*]

[a]*Powder Technology Laboratory, Insitute of Materials, Ecole polytechnique fédérale de Lausanne, Switzerland.*
[b]*Department of Radiology, University Hospital (CHUV) and University of Lausanne (UNIL), Switzerland. E-mail: marijanamionic@gmail.com.*
[c]*Center of Biomedical Imaging (CIBM), Lausanne, Switzerland*

Supporting Experimental Section

MRI. Imaging parameters for longitudinal relaxation times T_1: TR 12 s, TE 7.5 ms, slice thickness 3 mm, FOV 130 x 220 mm^2, matrix 266 x 448, RF excitation angle 90°, receiver bandwidth of 655 Hz/pixel, TI 23, 50, 75, 100, 125, 150, 250, 500, 1000, 5000, 10000 ms. Imaging parameters for transversal relaxation times T_2: TR 15 s, TE 7.5, 8.5, 10, 15, 20, 25, 30, 50, 100, 150, 400 ms, slice thickness 3 mm, FOV 130 x 220 mm^2, matrix 266 x 448, RF excitation angle 90°, receiver bandwidth of 655 Hz/pixel. The MRI signals of each suspension were determined using Image J and signal evolutions were analysed and fitted using Matlab (The MathWorks, Natick, MA, USA). The signal evolution S as function of TI and TE was fitted to derive the T1 and T2 of each γ-Fe_2O_3 nanoparticle suspension respectively, and is described as follows:

(1) $$\mathrm{S(TE)} = \mathrm{S(0)e}^{-\frac{\mathrm{TE}}{\mathrm{T_2}}} + \mathrm{C},$$

(2) $$\mathrm{S(TI)} = \mathrm{S(0)(1 - 2e}^{-\frac{\mathrm{TI}}{\mathrm{T_1}}}).$$

The T_1 and T_2 values as function of their γ-Fe_2O_3 nanoparticles concentration were subsequently fitted to obtain the relaxivities r_1 and r_2 described as:

(3) $$\frac{1}{\mathrm{T_{1,2}}} = \frac{1}{\mathrm{T_{1,2[0]}}} + \mathrm{r_{1,2}[\gamma - Fe_2O_3]}.$$

Cell culture. RWPE-1 cells were cultured in K-SFM containing human recombinant epidermal growth factor 1-53 and bovine pituitary extract (Gibco) supplemented with 1.5 % 10'000 U ml-1 Penicillin Streptomycin (Life Technologies); 22Rv1 and LnCaP cells were cultured in RPMI 1640 medium (ATCC) and PC3 cells in DMEM GlutaMAX with 25 mM high glucose and 1 mM sodium pyruvate (Gibco), all supplemented with 10 % fetal bovine serum (Life Technologies) and 1.5 % 10'000 U ml-1 Penicillin Streptomycin.

***In vitro* toxicity study.** The cell viability was assessed with the MTS test, as well as with APC-Annexin V and propidium iodide (PI). For the MTS test, 40'000 LnCaP or 15'000 RWPE-1 cells

per well were cultured in 96-well plates at 37 °C, and exposed to 100 µl of different concentrations of IONPs (0, 1, 5, 10, 50 and 100 μg_{Fe} ml^{-1}) for 1 h, 6 h and 24 h. Cells treated only with medium served as negative controls. After 24 h incubation, the supernatant of each well were removed. 100 µl of MTS solution (CellTiter 96® AQueous One Solution Cell Proliferation Assay from Promega, diluted 6 times in medium) was added to the cells. After 2 h incubation, the absorbance of the formazan product was measured with a microplate reader (Tecan Infinite M200) at a wavelength of 490 nm. For the Annexin V/PI assay, 600'000 22Rv.1, 350'000 LnCaP cells and 200'000 PC3 cells per well were cultured in 6 well plates, and exposed to 2.5 ml of different IONPs' concentrations (0, 5, 10, 50 and 100 μg_{Fe} ml^{-1}) for 24 h. Cells treated only with medium served as negative controls, and cells treated with 10 µM of staurosporine (diluted from 1 mM solution in DMSO, Sigma-Aldrich) for 20 h and 2 mM H_2O_2 (diluted from 3 % stock solution, Fluka) for 4 h were used as positive controls. After 24 h incubation, cells were washed once with PBS and detached from the wells with 0.05 % trypsin-EDTA. Cells were centrifuged and resuspended in 100 µl of Annexin V Binding Buffer (BioLegend). 12.5 µl of 1/25 APC-Annexin V solution (BioLegend) were added to the cells, which were incubated for 25 min at room temperature in the dark. 5 µl of PI and 100 µl of Annexin V Binding Buffer were then added. Samples were analyzed by flow cytometry (Accuri C6). APC-Annexin V was detected with with λ_{exc} = 640 nm and λ_{em} = 675 ± 12.5 nm and PI was detected with λ_{exc} = 488 nm and λ_{em} = 585 ± 20 nm. The cell viabilities are reported as cells negative for both Annexin V and PI. For both the MTS test and the Annexin V/PI assay, all experiments were performed in triplicates. Results are given as means (with standard deviations) of the values obtained in these triplicates.

In vitro **hyperthermia.** 300'000 22Rv.1, 70'000 LnCaP and 60'000 PC3 cells were suspended in 1 ml of growth medium and transferred into cryogenic vials (Thermo Scientific Nalgene). Cells were placed in a water bath at 44°C for 20 min or 40 min. Untreated cells, which served as negative controls, were placed in the incubator at 37°C for 20 min or 40 min. The cell viability was assessed (i) right after the heat treatment, and (ii) 15 h after the heat treatment, where cells were transferred in a 24 well plate and detached from the wells with 0.05 % trypsin-EDTA 15 h later. For both time points, cells were stained with Annexin V and PI, as described for the *in vitro* toxicity study, and analyzed by flow cytometry (Accuri C6).

Cellular uptake study after receptor blocking with FA. 200'000 22Rv.1 and 40'000 LnCaP cells per well were cultured in 12 well plates for the Prussian Blue staining. 600'000 22Rv.1 and 350'000 LnCaP cells per well were cultured in 6 well plates for the flow cytometry analysis. After 24 h, cells were exposed to 40 µM of FA for 15 min. After 15 min, cells were exposed to 1 ml of 100 μg_{Fe} ml^{-1} of FA-IONPs for 24 h. After 24 h, cells were stained with Prussian Blue as described in the main manuscript in the *cellular uptake study* section. The uptake of FA-IONPs was also evaluated by flow cytometry as described in the *in vitro toxicity study* section.

In vitro **MRI in cells (MRI of cells incubated with FA-IONPs).** We performed two different MRI:

1) 600'000 22Rv.1 per well were cultured in 6 well plates. Two wells per concentration were plated. After 24 h, cells were exposed to 2.5 ml of different concentrations of FA-IONPs (0, 10, 50 and 100 μg_{Fe} ml^{-1}) for 24 h. After 24 h, cells were washed with PBS and detached with 0.05 % trypsin-EDTA. Cells from two wells, which were incubated with the same concentration of FA-IONPs were put together in the same tube and centrifuged. The cell pellets were resuspended in 100 µl of 2 % agar. 1.5 ml eppendorf tubes were first filled with 600 µl of 2 % agar, then with the cell-aggar suspensions and finally with 1 ml of 2 % agar. 1.5 ml eppendorf tubes with only 2 % agar were used as controls.

MRI of 22Rv.1 cells. Imaging parameters for longitudinal relaxation times T_1: TR 12 s, TE 7.1 ms, slice thickness 5 mm, FOV 220 x 55 mm^2, matrix 384 x 96, RF excitation angle 90°, receiver bandwidth of 650 Hz/pixel, TI 23, 50, 75, 100, 125, 150, 250, 500, 1000, 5000, 10000 ms. Imaging parameters for transversal relaxation times T_2: TR 12 s, TE 7.1, 8.5, 10, 15, 20, 25, 30, 50, 100, 150, 400 ms, slice thickness 5 mm, FOV 220 x 55 mm^2, matrix 384 x 96, RF excitation angle 90°, receiver bandwidth of 655 Hz/pixel.

2) 350'000 LnCaP per well were cultured in 6 well plates. After 24 h, cells were exposed to 2.5 ml of different concentrations of FA-IONPs (0, 0.01 and and 0.1 μg_{Fe} ml^{-1}) for 24 h. After 24 h, cells were washed with PBS, detached with 0.05 % trypsin-EDTA and centrifuged. The cell pellets were resuspended in 50 µl of 2 % agar and transferred in 0.5 ml eppendorf tubes. The remaining empty space in the tubes was filled with 2 % agar. 0.5 ml eppendorf tubes filled with only 2 % agar were used as controls.

MRI of LnCaP cells. Imaging parameters for longitudinal relaxation times T_1: TR 12 s, TE 7.5 ms, slice thickness 3 mm, FOV 130 x 220 mm^2, matrix 266 x 448, RF excitation angle 90°, receiver bandwidth of 655 Hz/pixel, TI 23, 50, 75, 100, 125, 150, 250, 500, 1000, 5000, 10000 ms. Imaging parameters for transversal relaxation times T_2: TR 15 s, TE 7.5, 8.5, 10, 15, 20, 25, 30, 50, 100, 150, 400 ms, slice thickness 3 mm, FOV 130 x 220 mm^2, matrix 266 x 448, RF excitation angle 90°, receiver bandwidth of 655 Hz/pixel.

Supporting Figures and Tables

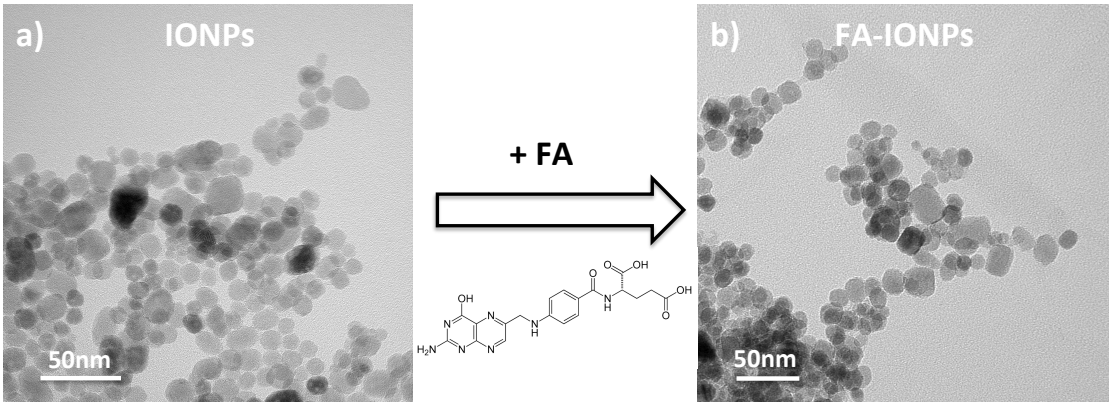

Fig. S1: Representative TEM micrographs of uncoated IONPs (a) and FA-IONPs (b). The chemical structure of FA is given in the center of the figure.

Table S1: Atomic percentages of Fe, O, C and N obtained by XPS in uncoated IONPs and FA-IONPs.

Atomic %	**Fe 2p**	**O 1s**	**C 1s**	**N 1s**
IONPs	32.0	50.6	17.3	-
PLP-IONPs-pH7	10.3	28.7	48.0	13.1

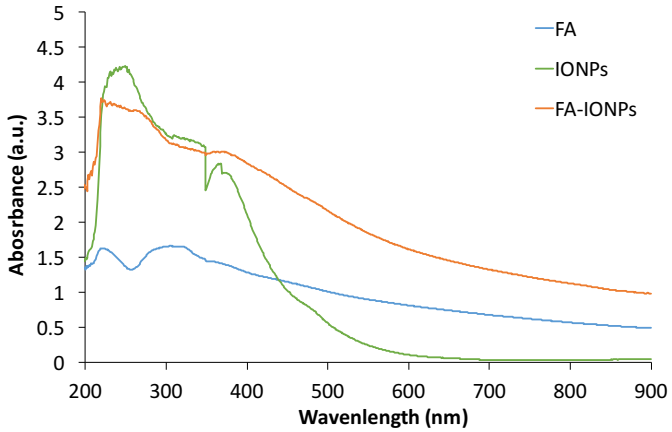

Fig. S2: Absorbance of FA, IONPs and FA-IONPs measured by UV-visible spectroscopy.

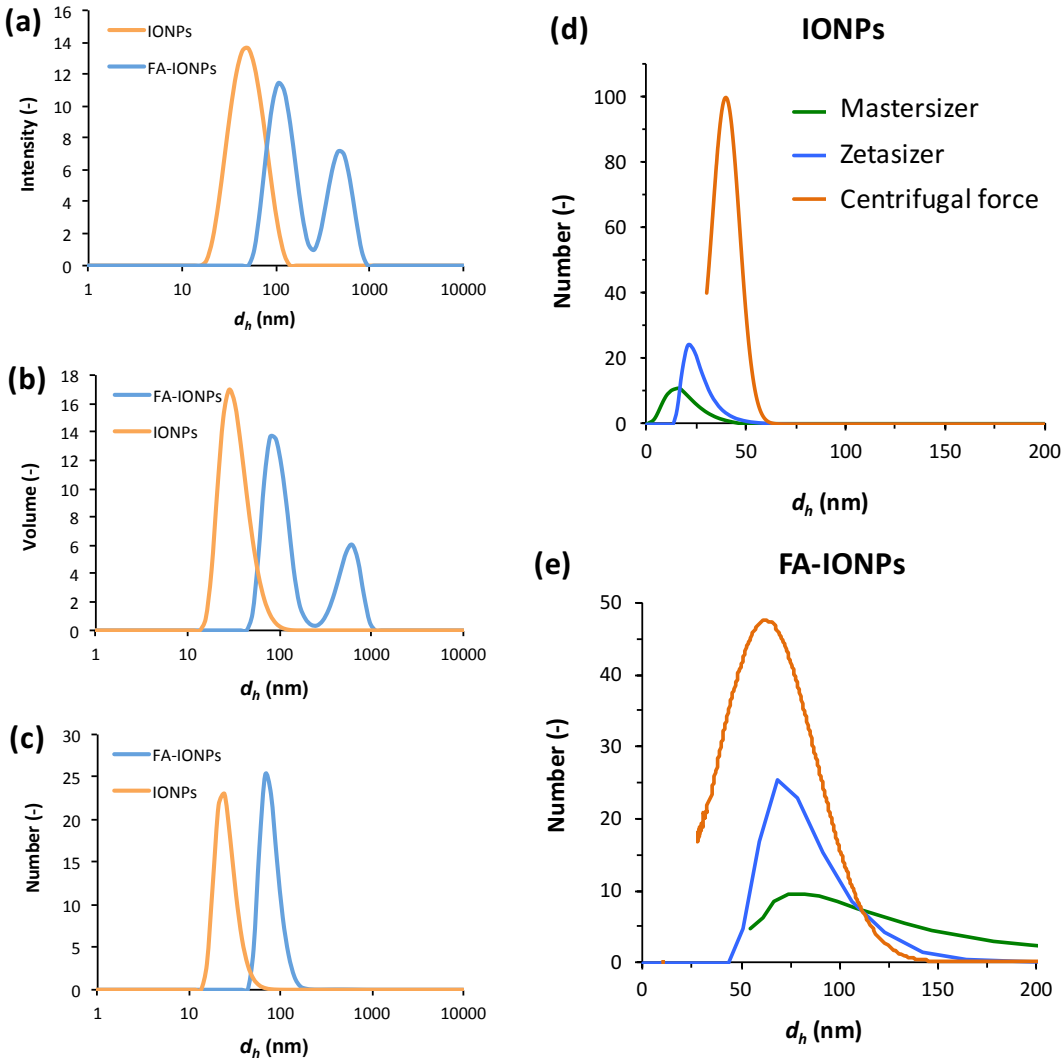

Fig. S3: (a-c) Distribution of the hydrodynamic diameter, d_h, of IONPs and FA-IONPs given in intensity (a), volume (b) and number (c) measured by DLS with the Zetasizer. (d-e) Number weighted distribution of the hydrodynamic diameter, d_h, of IONPs (d) and FA-IONPs (e) measured in H_2O (pH ~6) by dynamic light scattering (DLS) with the Mastersizer (Malvern Instruments) or with the Zetasizer (Malvern Instruments), or by centrifugal force (CPS Instruments).

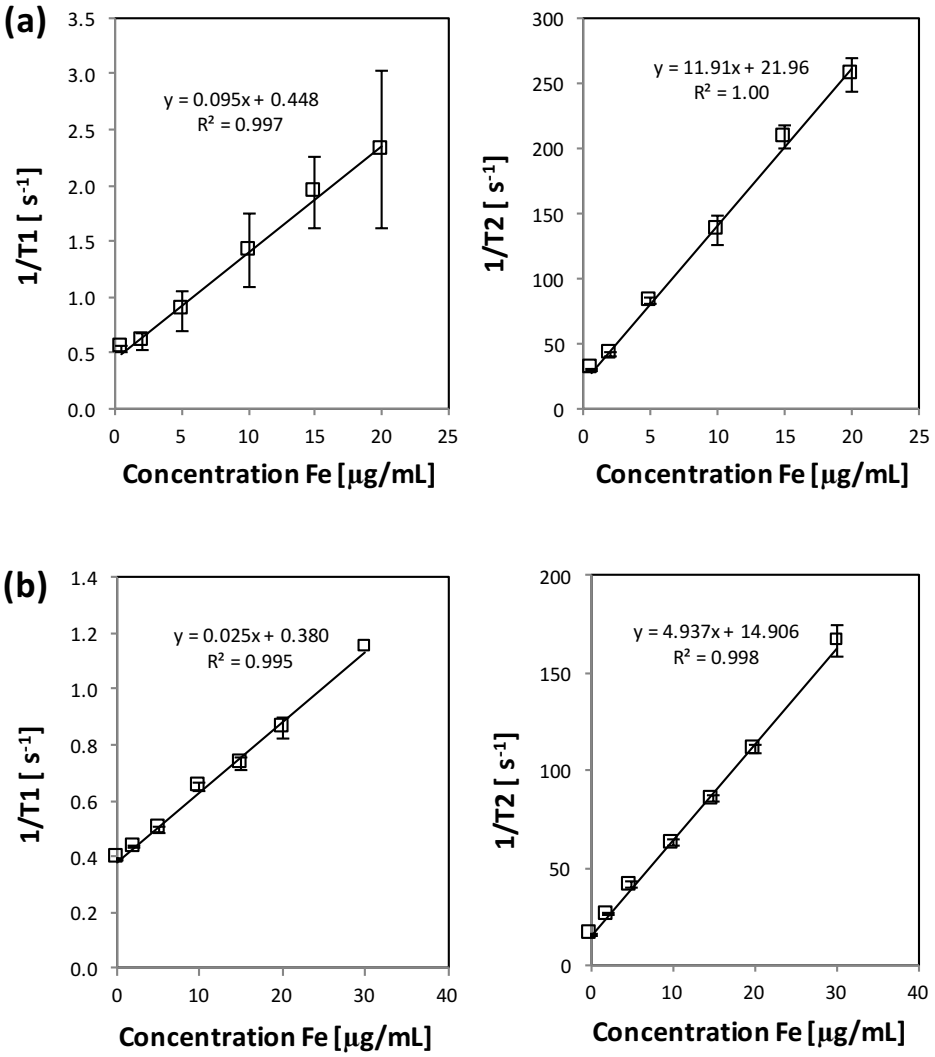

Fig. S4: 1/T1 and 1/T2 as a function of the Fe concentration measured at 3 T for uncoated IONPs and FA-IONPs.

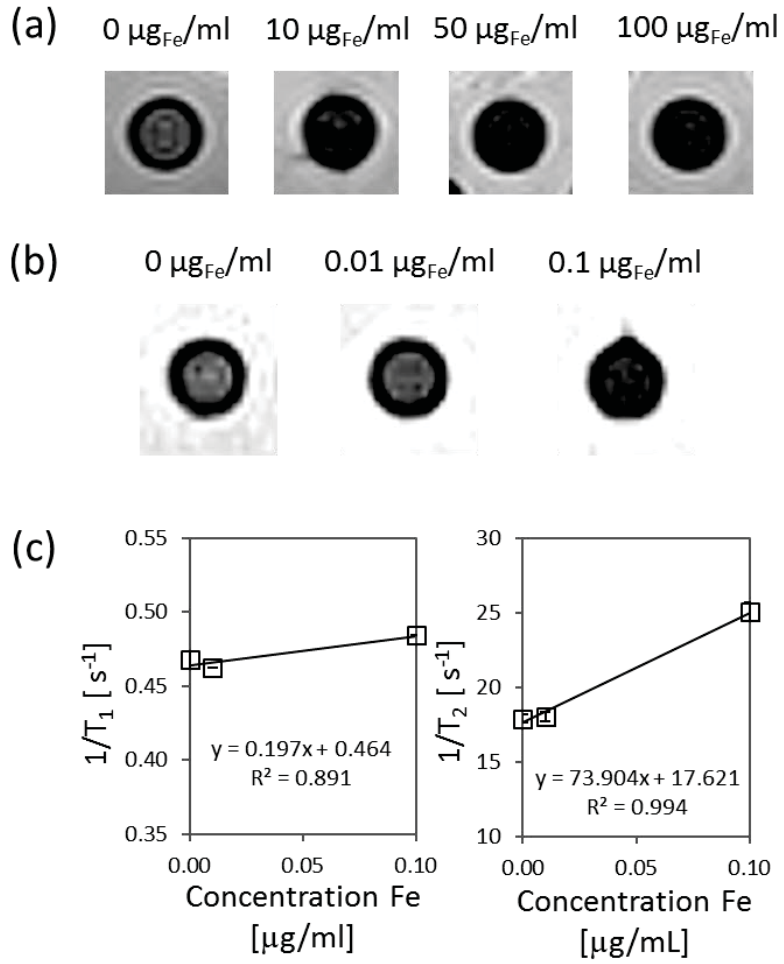

Fig. S5: (a) MR images of 22Rv.1 cells after incubation for 24h without (0 μg_{Fe}/ml) and with FA-IONPs at concentrations of 10, 50 and 100 μg_{Fe}/ml. (b) MR images of LnCaP cells after incubation for 24h without (0 μg_{Fe}/ml) and with FA-IONPs at concentrations of 0.01 and 0.1 μg_{Fe}/ml. (c) $1/T_1$ and $1/T_2$ as a function of the Fe concentration measured at 3 T in LnCaP cells after 24h incubation without (0 μg_{Fe}/ml) and with FA-IONPs at concentrations of 0.01 and 0.1 μg_{Fe}/ml.

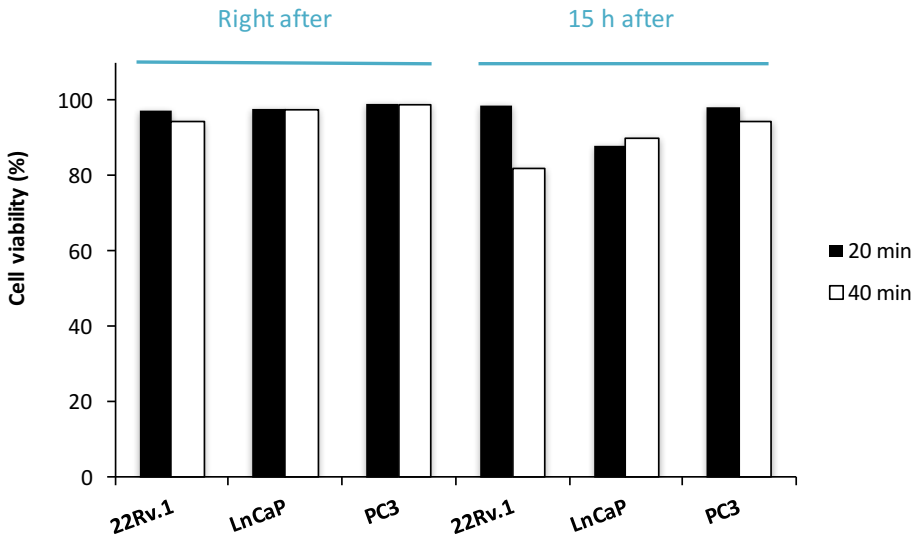

Fig. S6: Viability of 22Rv.1, LnCaP and PC3 cells treated for 20 min or 40 min at 44°C in a water bath. The cell viability was measured with the Annexin V/PI assay right after or 15 h after the heat treatment. The cell viabilities are the number of viable cells (negative for both Annexin V and PI), which were subjected to heat, normalized with the number of viable cells without heat treatment.

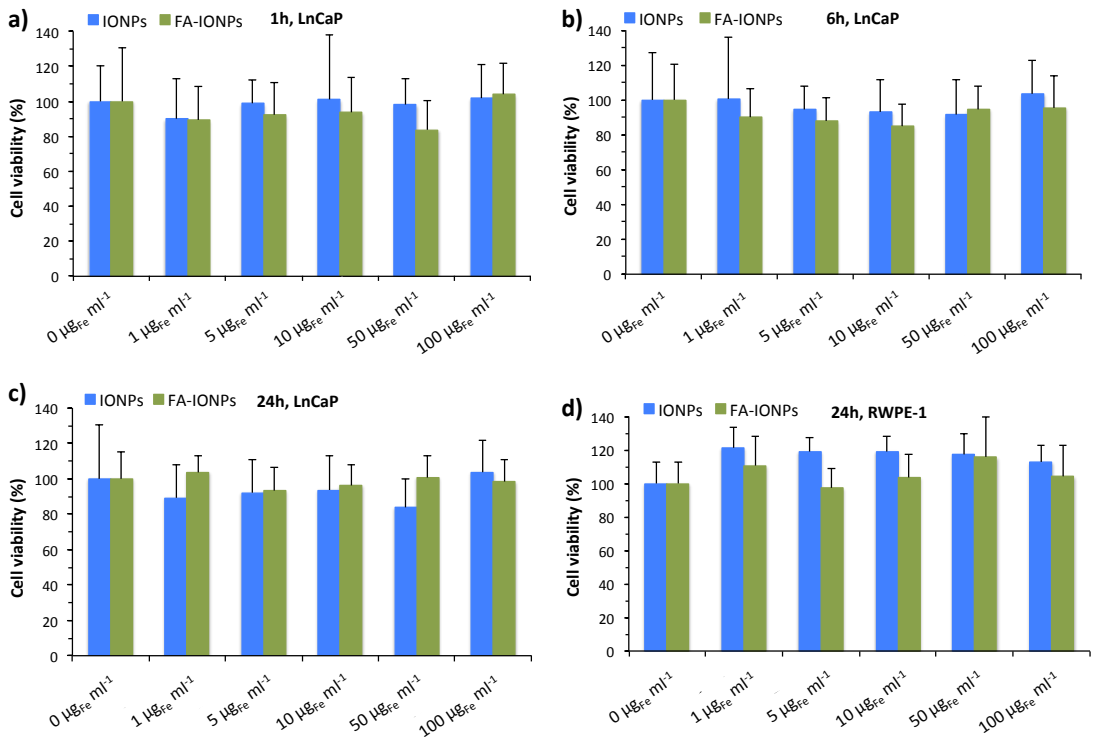

Fig. S7: Viability of LnCaP (a, b and c) and RWPE-1 (d) cells incubated for 1 h (a), 6 h (b) and 24 h (c, d) with different concentrations (0, 1, 5, 10, 50 and 100 μg_{Fe} ml^{-1}) of uncoated IONPs and FA-IONPs measured with the MTS test. The cell viabilities are the percentages obtained from the absorbance of cells treated with IONPs normalized with the absorbance of cells without IONPs (0 μg_{Fe} ml^{-1}).

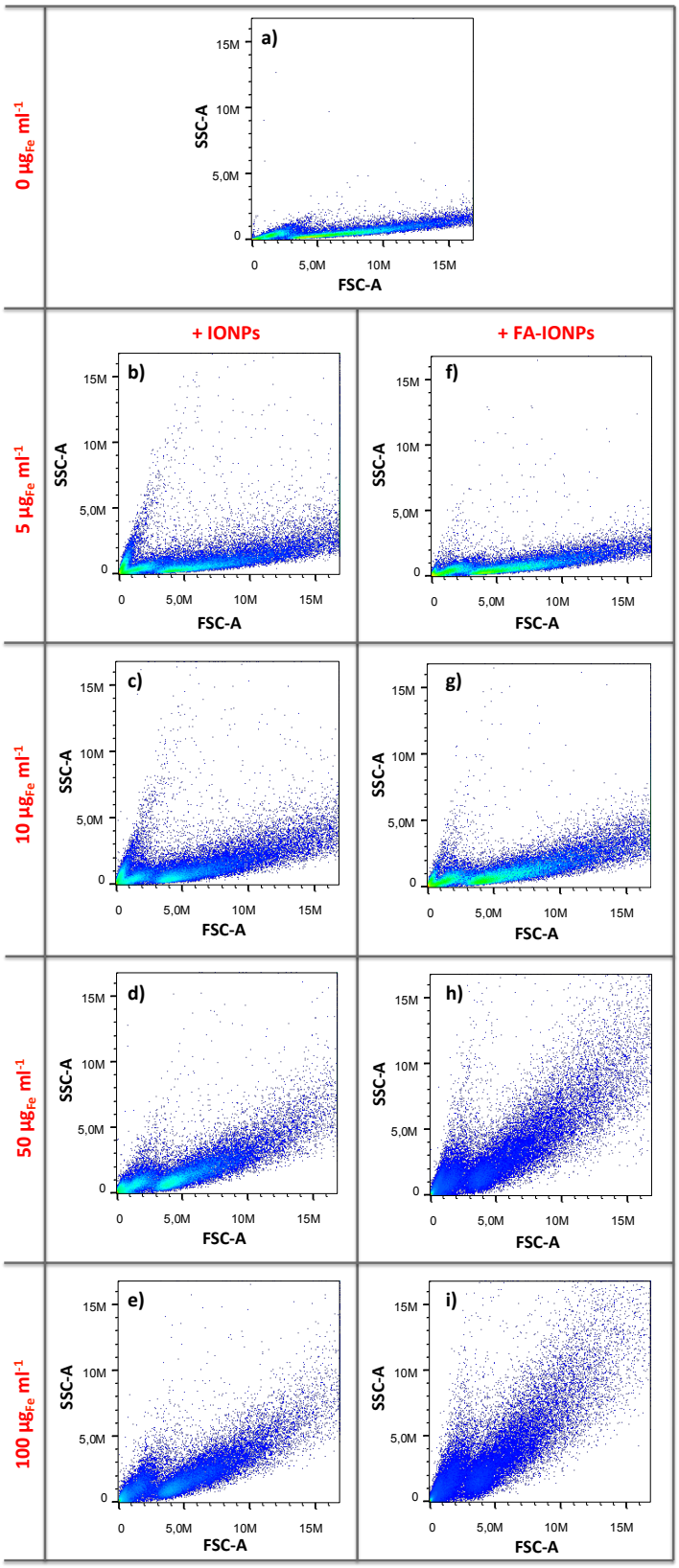

Fig. S8: Forward scatter (FSC) *vs* side scatter (SSC) plot of 22Rv.1 cells without IONPs (0 μg_{Fe} ml^{-1}; a) or treated for 24 h with different concentrations (5, 10, 50 and 100 μg_{Fe} ml^{-1}) of uncoated IONPs (b, c, d, e) and FA-IONPs (f, g, h, i). SSC-A and FSC-A = area of the side and forward light scatter pulse. Data from a selected experiment.

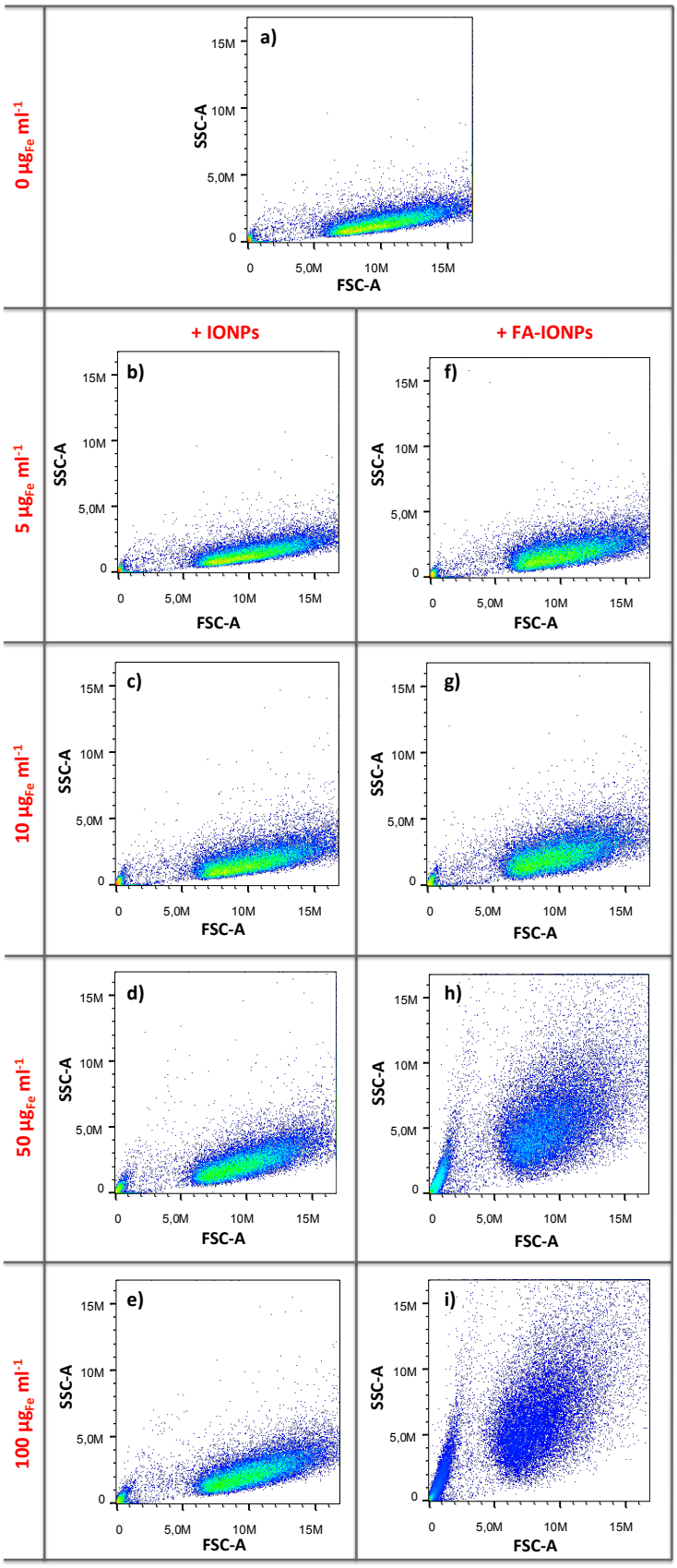

Fig. S9: Forward scatter (FSC) *vs* side scatter (SSC) plot of PC3 cells without IONPs (0 µg$_{Fe}$ ml^{-1}; a) or treated for 24 h with different concentrations (5, 10, 50 and 100 µg$_{Fe}$ ml^{-1}) of uncoated IONPs (b, c, d, e) and FA-IONPs (f, g, h, i). SSC-A and FSC-A = area of the side and forward light scatter pulse. Data from a selected experiment.

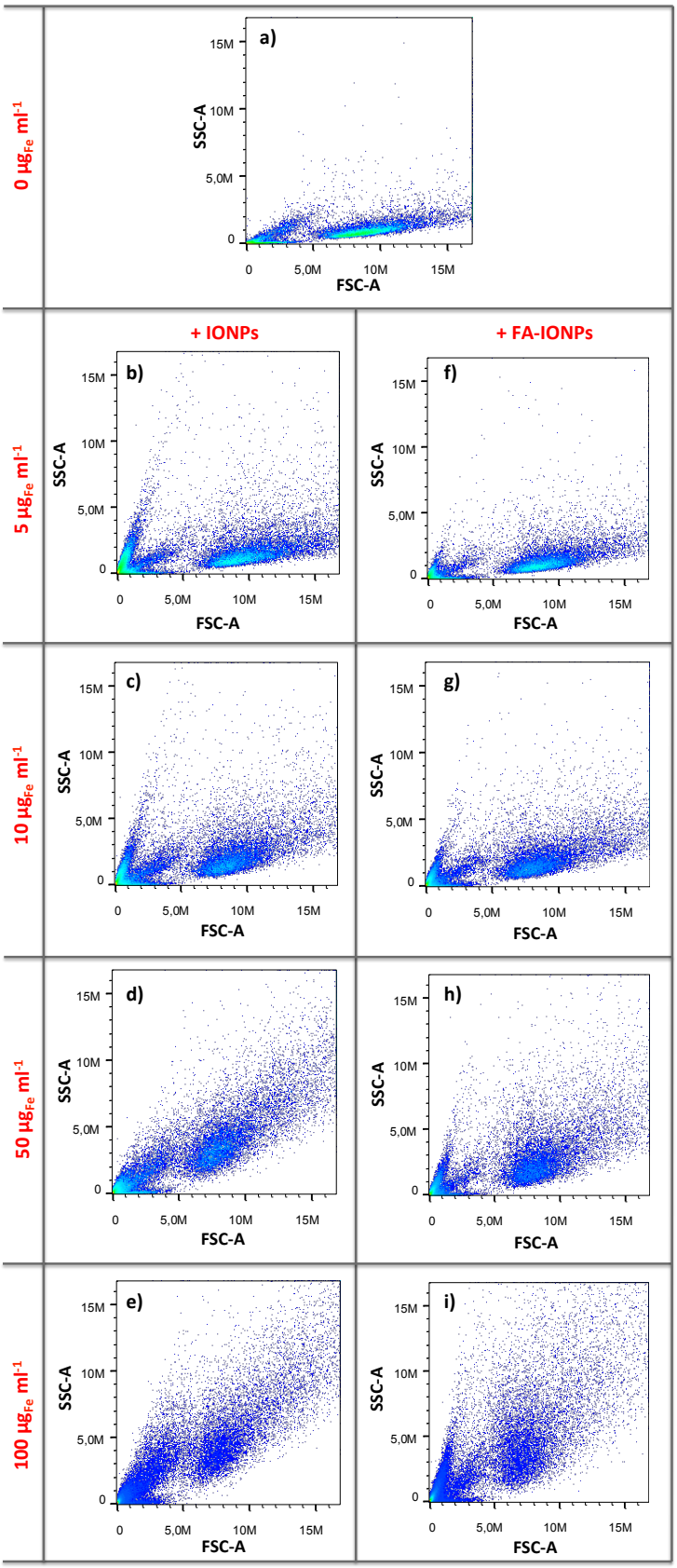

Fig. S10: Forward scatter (FSC) *vs* side scatter (SSC) plot of LnCaP cells without IONPs (0 µg$_{Fe}$ ml^{-1}; a) or treated for 24 h with different concentrations (5, 10, 50 and 100 µg$_{Fe}$ ml^{-1}) of uncoated IONPs (b, c, d, e) and FA-IONPs (f, g, h, i). SSC-A and FSC-A = area of the side and forward light scatter pulse. Data from a selected experiment.

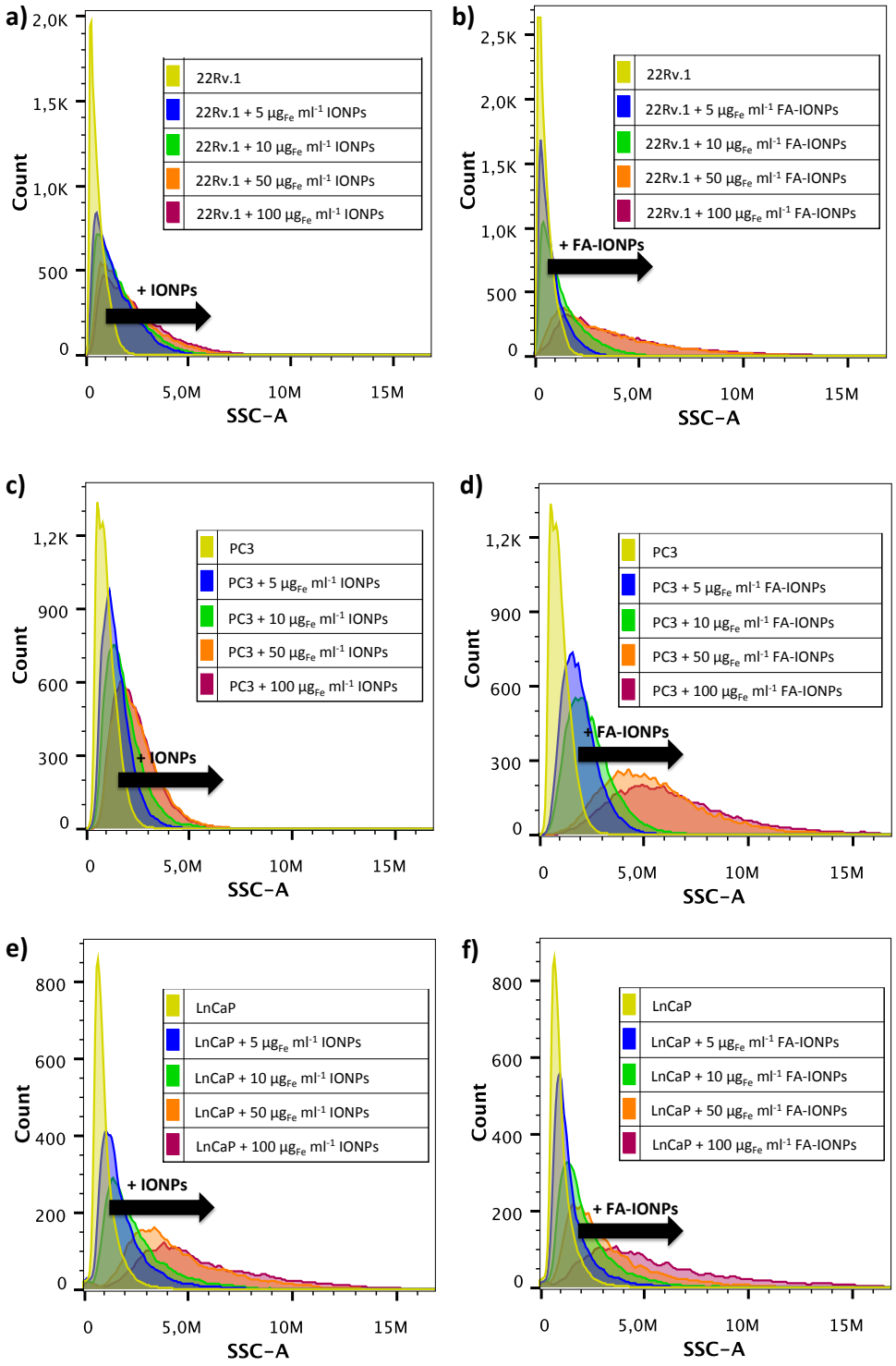

Fig. S11: Counts *vs* area of the side scatter (SSC-A) plot of 22Rv.1 (a, b), PC3 (c, d) and LnCaP cells (e, f) treated for 24 h with different concentrations (5, 10, 50 and 100 µg$_{Fe}$ ml^{-1}) of uncoated IONPs (a, c, e) and FA-IONPs (b, d, f). Data from experiments selected in Fig. S4, S5 and S6.

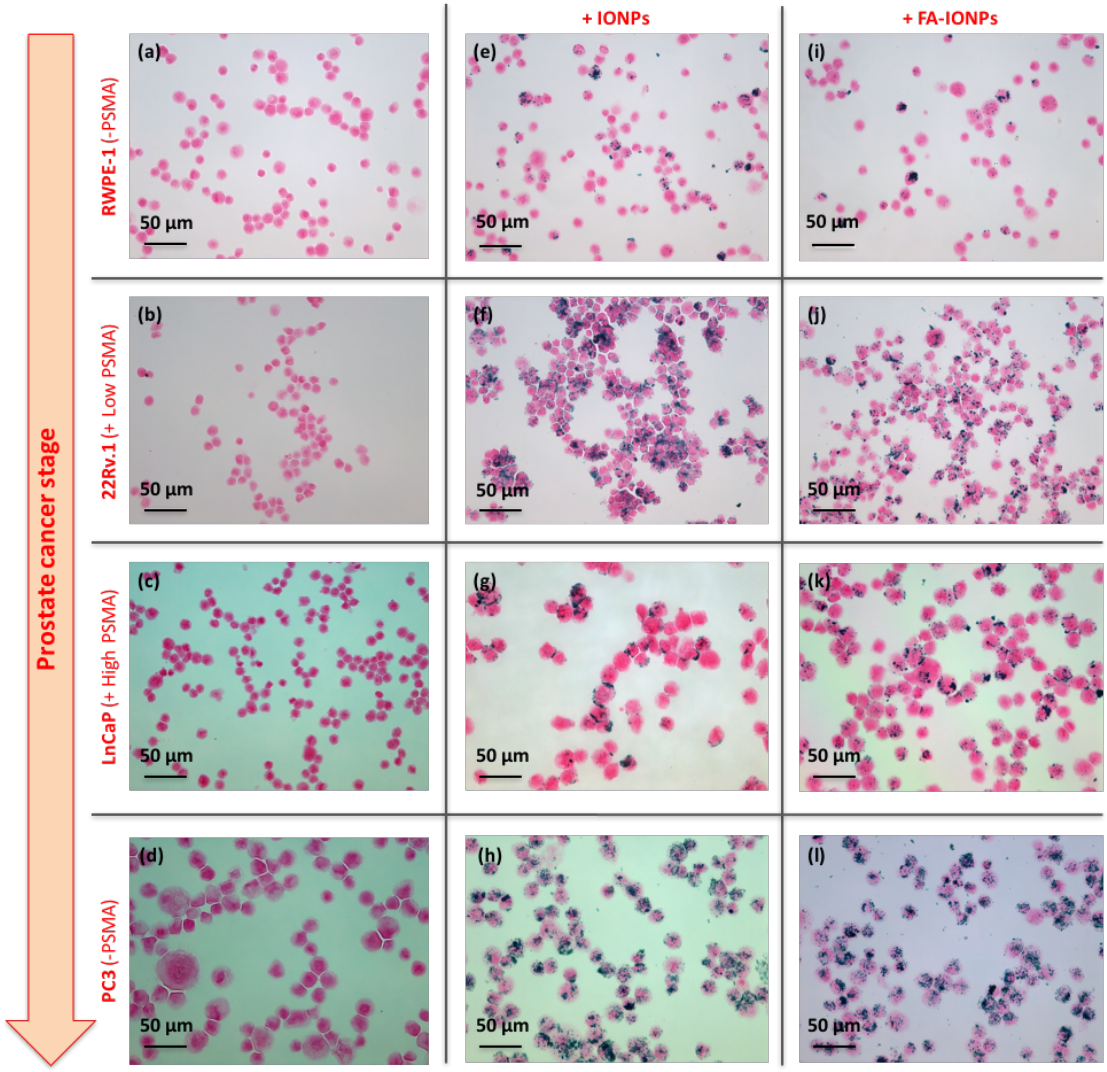

Fig. S12: Transmitted light microscopy micrograph of RWPE-1 (a, e, i), 22Rv.1 (b, f, j), LnCaP (c, g, k) and PC3 (d, h, l) without IONPs (a, b, c, d) or incubated for 24 h with 10 μg_{Fe} ml^{-1} of uncoated IONPs (e, f, g, h) or FA-IONPs (i, j, k, l). Cells were stained with Nuclear Fast Red (light pink staining) and Prussian Blue (dark blue staining), highlighting the cellular cytoplasm and the iron, respectively.

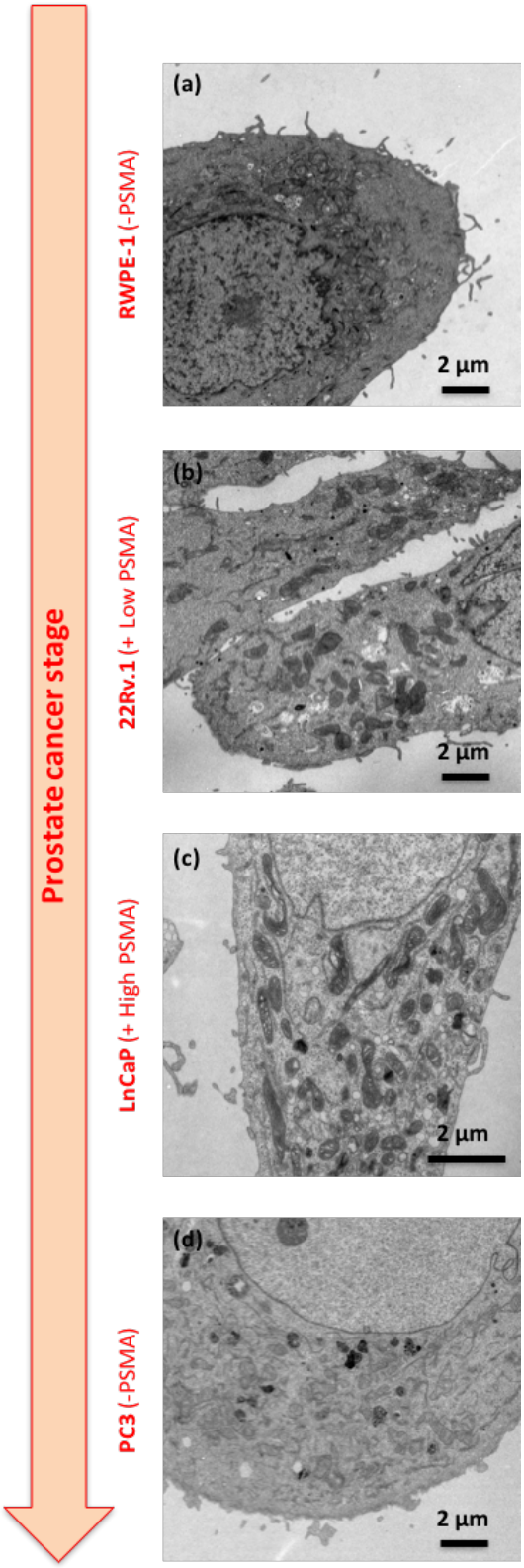

Fig. S13: TEM micrographs of 50 nm-thick sections of RWPE-1 (a), 22Rv.1 (b), LnCaP (c) and PC3 (d) cells. The presence and the absence of PSMA in each cell lines are indicated by +PSMA or –PSMA, respectively.

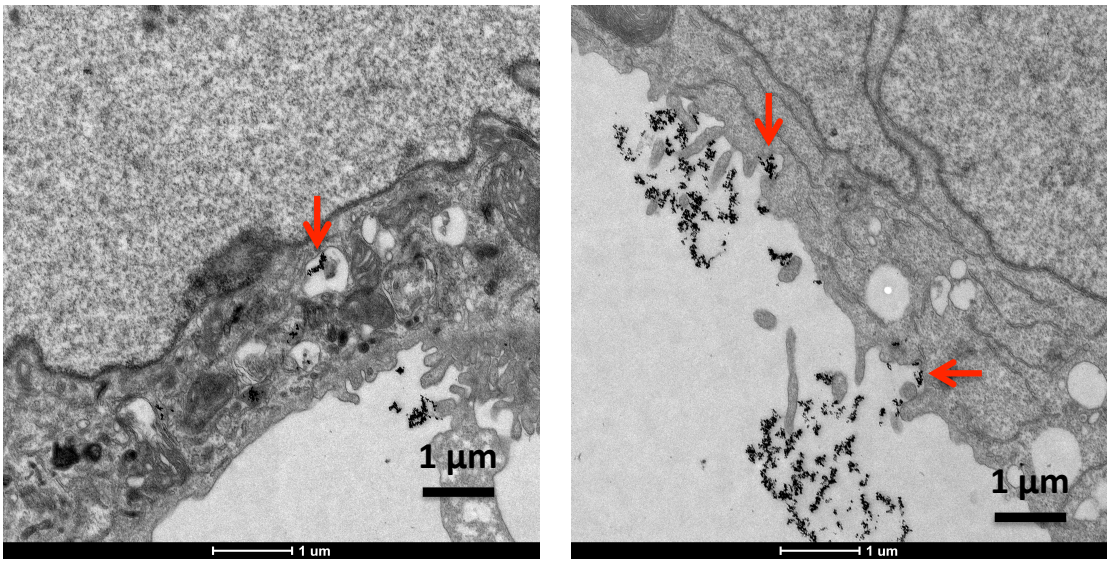

Fig. S14: TEM micrographs of 50 nm-thick sections of LnCaP cells incubated for 1 h with 100 μg_{Fe} ml^{-1} FA-IONPs which show FA-IONPs already inside cells (left panel) and FA-IONPs within the engulfed cell membrane (right panel). Red arrows indicate FA-IONPs inside cells or inside cell membrane invaginations ready to be taken up.