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Antenna-type radiofrequency generator in nanoparticle-mediated hyperthermia

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Materials and methods

Materials

Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), sodium hydroxide, gold (III) chloride tri-hydrate (HAuCl4•3H2O, hexadecyltrimethylammonium bromide (CTAB), potassium borohydride (KBH4), silver nitrate ($AgNO_3$) were supplied from Sigma-Aldrich (Germany). tetramethylammonium hydroxide solution (TMAOH, 25 wt. % in H_2O), sodium hydroxide were purchased from MERCK, (Germany). Also, Tri-sodium citrate (SAFC, USA), L-ascorbic Acid (SAFC, USA), foetal bovine serum (FBS, Serva, Israel), WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2h-tetrazolium, Roche, Germany], phosphate-buffered saline (PBS, Sigma, Germany), Hoechst dye 33342 (Thermo Scientific, USA), propidium iodide (PI) (Sigma-Aldrich, Germany), penicillin/streptomycin (Life Technologies, USA) and DNAse-free RNAs (Thermo Scientific, USA) were supplied before experiments. All chemicals were of analytical grade and were used without further purification. Aqueous solutions were prepared using deionized (DI) water that had been produced by Millipore Milli-Q Plus water purification system. MCF-7 and L-929 cells were supplied from the Department of Molecular Biology and Genetics (Bilkent University, Turkey) and the Sap Institute (Ankara, Turkey) respectively.

Synthesis of nanoparticles

Synthesis of SPIONs

SPIONs were prepared according to the co-precipitation method reported by Kang et *al.* [1] Briefly, 5 ml of $FeCl_2 \cdot 4H_2O$ (0.0994 g) and $FeCl_3 \cdot 6H_2O$ (0.2703 g) solution at a Fe^{+2}/Fe^{+3} molar ratio of 0.5 was prepared and purged with N_2 for 10 min to remove dissolved oxygen. In an aqua regia treated three-neck round-bottom flask, 50 ml of 1 M NaOH solution was purged with N_2 at a high stirring rate for 20 min. Subsequently, the Fe solution was added drop wise to the NaOH solution at a high stirring rate (2000 rpm) in N_2 atmosphere. Efficient addition and mixing of solutions was maintained to prevent aggregation. The color of the solution initially changed to dark brown and finally to dark black. The reaction was terminated after 30 min. SPIONs were removed from the basic solution by applying a strong magnet and were then

washed at least three times with distilled water to remove the non-magnetic components. Finally, SPIONs were dispersed in 0.1 M TMAOH solution to stabilize and prevent oxidation.

Synthesis of Au@SPIONs

Au@SPIONs were prepared according to the methods reported by Pham et al. with minor modifications.[2] 0.5 ml of a TMAOH-stabilized SPIONs solution was dispersed in 100 ml of 0.01 M sodium citrate solution and the suspension was sonicated for 20 min to exchange OH^- ions with citrate ions. Subsequently, the solution was heated to boiling point and 0.42 ml of 10 mM $HAuCl_4$ was added drop wise under continuous stirring. The reaction was terminated after 20 min. To remove AuNPs from Au@SPIONs, a strong magnet was applied and the nanoparticle solution was washed three times with distilled water.

Synthesis of AuNPs

AuNPs were prepared using a standard citrate reduction method as reported by Turkevitch.[3] In brief, 50-ml aqueous solutions of 1 mM $HAuCl_4$ were heated to boiling point and then Au^{+3} ions were reduced by addition of 16.5 ml of 0.01 M tri-sodium citrate solution under vigorous stirring. Reduction of gold ions was sustained for 10 min to achieve complete reduction of Au^{3+} to Au^0. Final dark red solution of AuNPs was stored at room temperature for further use.

Synthesis of AuNRs

AuNRs were synthesized according to the method developed by El-Sayed group.[4]. Seed solutions and growth solutions were prepared separately and AuNRs were synthesized with the desired aspect ratio of approximately 3. Initially, the seed solution was prepared by mixing 2.5 ml of 1 mM $HAuCl_4$, 2 ml of 0.5 M CTAB and 0.15 ml of 0.02 M KBH_4 under vigorous stirring. Synthesis of the seed solution was indicated by a brownish red color. Subsequently, 18 µl of the freshly prepared seed solution was added to the growth solution containing 2.5 ml of 1 mM $HAuCl_4$, 2 ml of 0.5 M CTAB, 0.5 ml of 1 mM silver nitrate and 0.25 ml of 0.1 M L-ascorbic acid under magnetic stirring. The synthesis reaction was sustained for 20 min to ensure maximum conversion. The resulting solution had a pinkish red color and was washed

with DI water and was centrifuged two times at 10000 rpm for 20 min to remove excess CTAB and un AuNPs.

Cell viability tests: The effects of nanoparticle type and RF exposure

MCF-7 and L-929 cells were plated 96-well-plates at a density of 5×10^3 cells/well for all measurements of cytotoxicity. Initially, cell viability of each nanoparticle type (SPIONs, Au@SPIONs and AuNPs) was investigated at nanoparticle concentrations of 5–30 μg/ml. The cells were incubated in a CO_2 incubator with 95% humidity for 24 h after addition of nanoparticles. Subsequently, 15 μg of WST-1(Cell Proliferation Reagent) was added to each well and incubation was continued for another 4 hours. The well-plates were then read using a standard ELISA microplate reader (Biotek, Gene 5 power wave XS2, USA) at 440 nm and 630 nm. In subsequent experiments, the cells were incubated for 4 hours and were then exposed to various RF powers for 30 min. The cells were treated with 5–30 μg/ml of AuNP, Au@SPION or SPION dispersions and then were induced by RF exposure at 80–180 W for 30 min and then were incubated at 37°C for 12 hours. Following incubation, 15 μg of WST-1 was added to each well and the cells were incubated for 4 hours. The well-plates were read using an ELISA reader. All RF exposure experiments were repeated at least three times.

Apoptosis and necrosis tests: The effects of nanoparticle type and RF exposure

Double staining with Hoechst 33342 dye and PI was applied to quantify the number of apoptotic and necrotic cells in culture on the basis of cell nuclei scores, respectively. MCF-7 and L-929 cells (1×10^4 cells/well) were grown in DMEM-F12 medium supplemented with glutamine, 10% fetal calf serum and 1% penicillin–streptomycin at 37°C in a 5% CO_2 humidified atmosphere in 48-well-plates. The first group of MCF-7 and L-929 cells was treated with nanoparticles (SPION, AuNPs and Au@SPION) at final nanoparticle concentrations of 5, 15 and 30 μg/ml for 24 hours. Simultaneously, as control groups, MCF-7 and L-929 cells were treated with fresh

cell culture medium without any nanoparticle dispersion. The second group of cells was treated with SPIONs, Au@SPIONs and AuNPs at concentrations of 5, 15 and 30 μg/ml in cell culture medium for approximately 4 hours for various RF powers exposures. The cells were induced with 80–180 W RF power at 144.015 MHz for 30 min and were kept into the incubator at 37°C for 12 hours. Both attached and non-attached cells (co-cultured cell groups) were collected at the end of the experiments. The cell culture medium was washed with Phosphate Buffered Saline solution (PBS) and the cells were stained with Hoechst 33342 dye (2 μg/ml), PI (2 μg/ml) and DNAse-free RNAse (100 μg/ml) for 15 min at room temperature. Subsequently, 10–50 μl of cell suspensions were smeared onto glass slides for fluorescence microscope examination. Nuclei of normal cells were light blue stained with blue fluorescence of low intensity, whereas apoptotic cells were stained with strong blue fluorescence by the Hoechst 33342 dye. According to microscopic observations, Apoptotic cells were experienced also identified by morphological changes in the nuclei, including nuclear fragmentation and chromatin condensation. [5,6] Necrotic cells' nuclei were stained red with PI dye, which can diffuse into cell membranes, whereas non-necrotic cells block PI dye diffusion because of greater plasma membrane integrity.[7] Apoptotic and necrotic cells were counted in 10 randomly selected microscopic fields at 40× magnification using a DMI600 fluorescence inverted microscope (Leica, Germany) equipped with DAPI and FITC filters and which were compared with healthy cells.

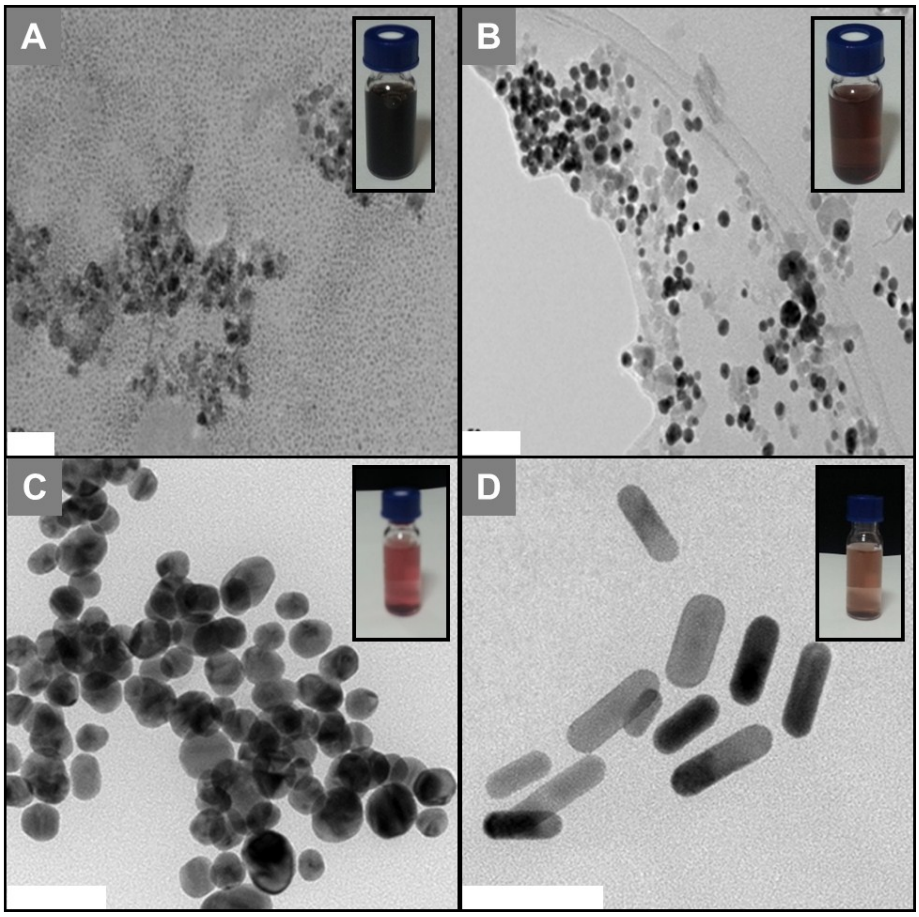

Fig. S1. TEM images of synthesized nanoparticles A) SPIONs, B) Au@SPIONs, C) AuNPs and D) AuNRs. Insets show photography images of colloidal nanoparticle solution. Scale bars are 50 nm.

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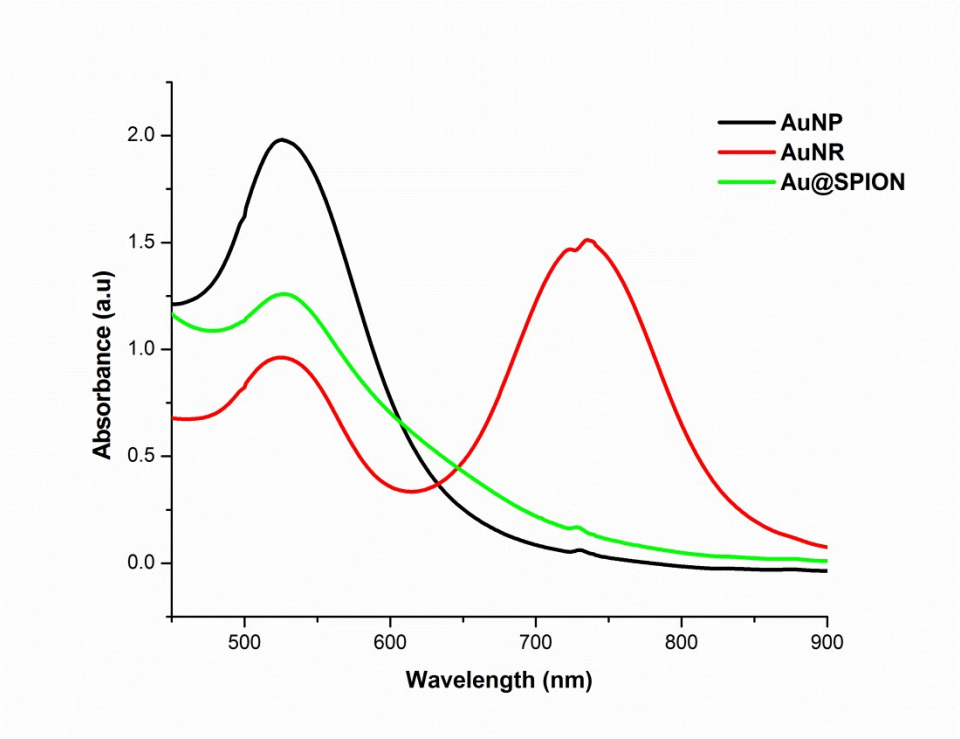

Fig. S2. UV–vis spectra of synthesized nanoparticles: Au@SPIONs, AuNPs and AuNRs.

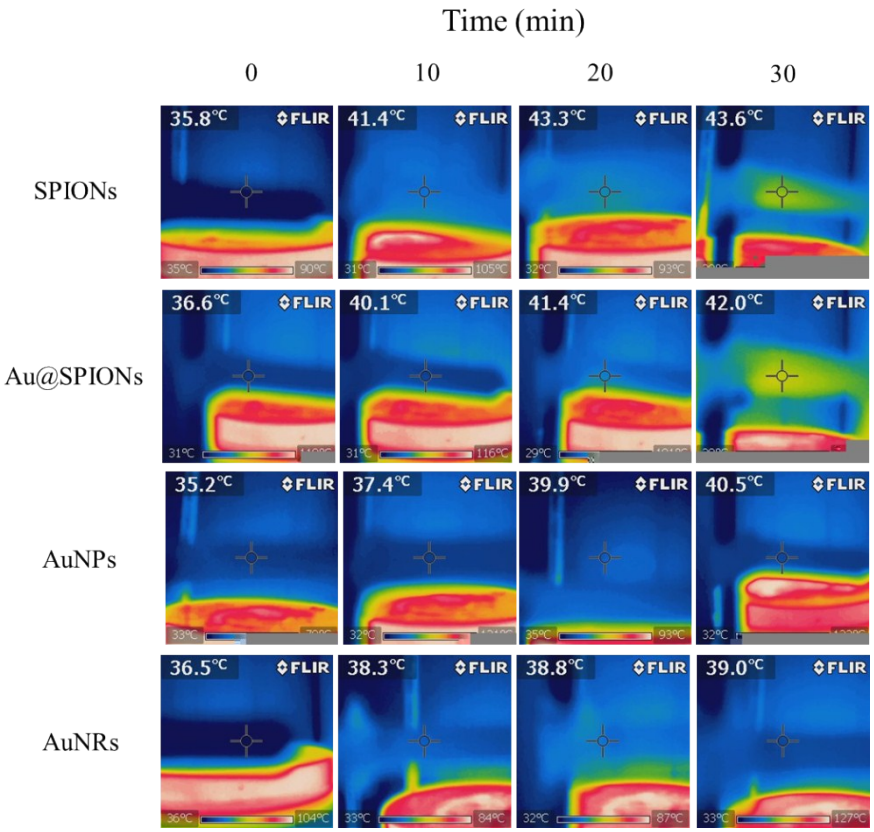

Fig. S3. Time-lapse images captured by infrared thermal camera (FLIR i5) with 30 μg/ml of nanoparticle concentration and 180 W RF power.

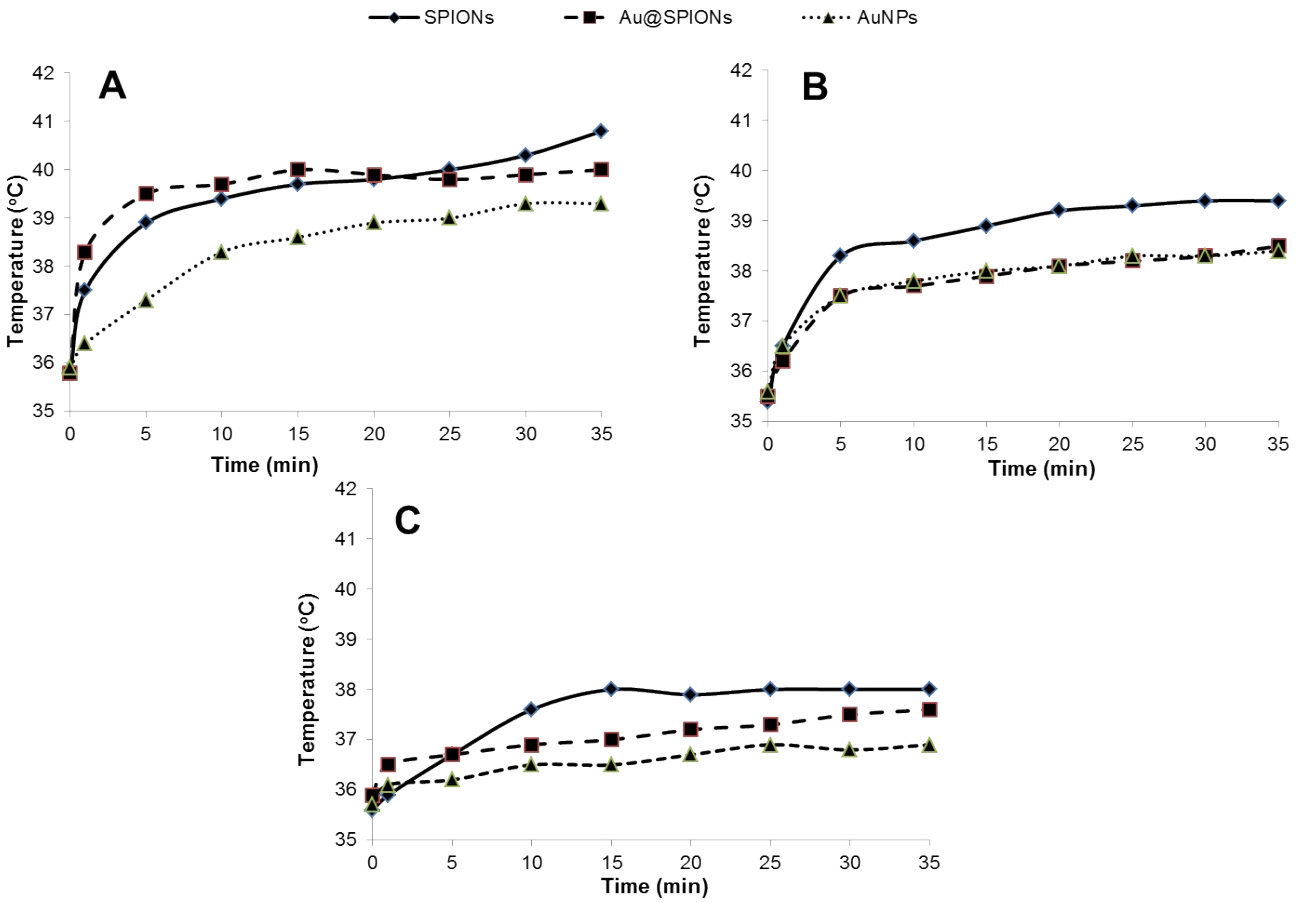

Fig. S4. Temperature rise curves in the presence of 15 µg/ml of nanoparticle concentration with A) 180 W, B) 120 W and C) 80 W RF powers.

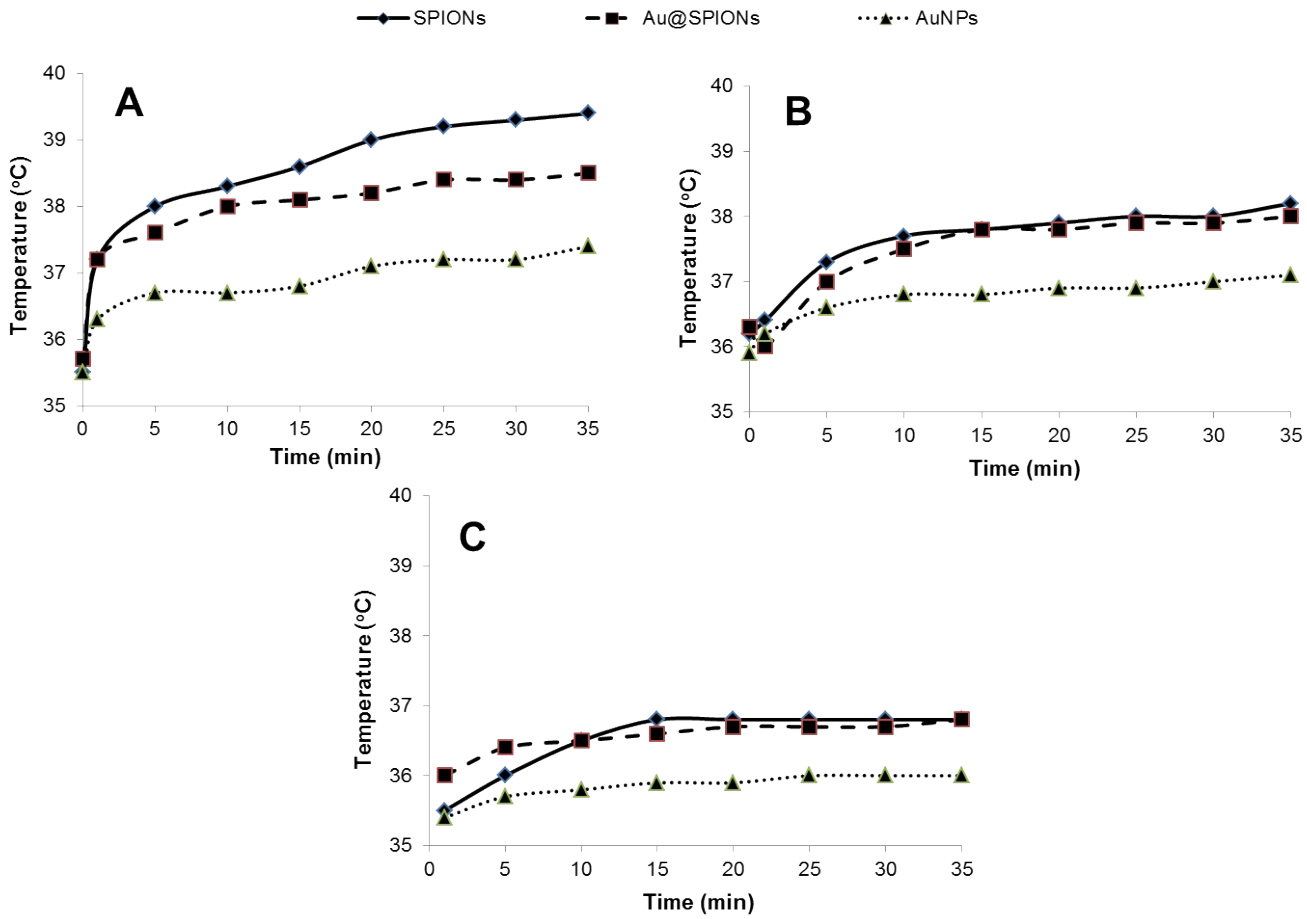

Fig. S5. Temperature rise curves in the presence of 5 µg/ml of nanoparticle concentration with A) 180 W, B) 120 W and C) 80 W RF powers.

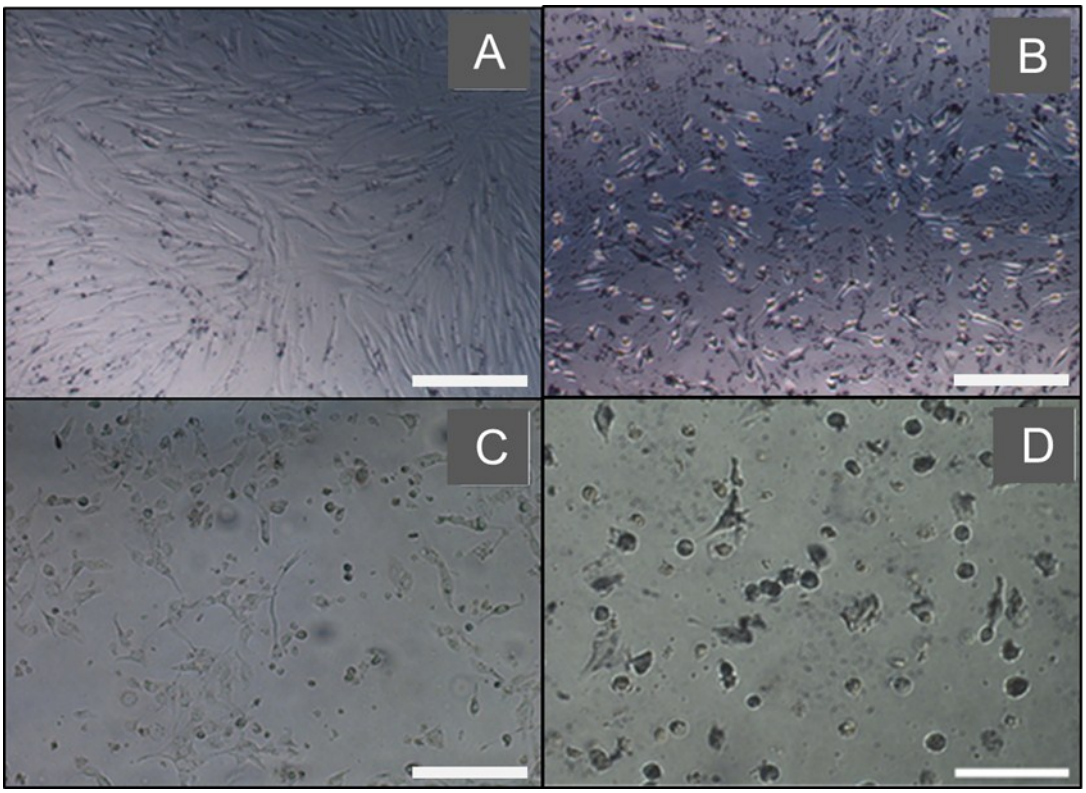

Fig. S6. Light microscopy images of L-929 and MCF-7 cells.

Fibroblast L-929 cells in the presence of 30 μg/ml SPIONs before (A) and after (B) 180 W RF exposure; MCF-7 cells in the presence of 30 μg/ml SPIONs before (C) and after (D) 180 W RF exposure. The images were taken at 100× magnification using a Leica DMI6000 model inverted microscope. Bars represent 200 μm. SPIONs can be observed as black dots in all cell culture images.

References

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