Development of Multifunctional Hyaluronan Coated Nanoparticles for Imaging and Drug Delivery to Cancer Cells

Supporting Information

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Experimental procedures and methods

1. Synthesis of SPION

To a solution of dextran (10 g) in 0.22 μm filter filtered deionized water (23 ml) was added $FeCl_3.6H_2O$ (0.7g, 2.6 mmol) and the solution was cooled in an ice bath (ice/water). To improve the magnetic properties of the iron oxide nanoparticles, the mixture was stirred under Argon or nitrogen for 2 hours to eliminate oxygen from the reaction flask. A freshly prepared 0.2 μm filtered aqueous solution containing $FeCl_2.4H_2O$ (0.28 g, 1.4 mmol) in water (1 ml) was added to the cooled solution. Chilled 30% NH_4OH (1 ml) was then added dropwise while stirring rapidly. The black suspension was brought to 70-80^oC over a 1 hr period, and maintained within this interval for additional 90 min. After being cooled to room temperature, the suspension was dialyzed (MWCO 14,000) against distilled water (20 L bucket, 4 changes). The excess ammonium hydroxide, ammonium chloride, and dextran were further removed by ultrafiltration (MWCO 100,000). After 4 washes, the suspension was concentrated to ~ 75 ml, collected and subjected to centrifugation to remove any large particulates. Cross-linking the dextran coating on the surface of SPION was achieved by adding an aqueous sodium hydroxide solution (5 M, 20 ml) and epichlorohydrin (10 ml). The mixture was stirred rapidly for 24 hr at room temperature to allow the interaction between the organic epichlorohydrin and the aqueous phase containing SPION. The excess epichlorohydrin was removed by dialysis against distilled water (MWCO 14,000) in a 20 L bucket (6 changes). The solution was concentrated to ~ 100 ml by ultrafiltration. The introduction of primary amino groups to the surface of the cross-linked dextran coated SPION was achieved by adding 30% NH_4OH (20 ml) and stirring the mixture at 37^oC for 36 hr. The colloidal solution was dialyzed (MWCO 14,000) against distilled water (20

L bucket, 4 changes), and then concentrated to 50 ml by ultrafiltration (MWCO 100,000). The FITC-SPION was prepared by adding FITC (5 mg), dissolved in DMSO (2 ml), to an aqueous solution of amine-functionalized SPION (7 mg/ml, 10 ml) and stirring the mixture in the dark at room temperature for 48 hr. The mixture was purified by dialysis (MWCO 14,000) and ultrafiltration (MWCO 100,000) to remove the unreacted FITC. The percentage of FITC labeling (w/w) was determined by UV-vis spectroscopy.

2. Synthesis of HA-SPION

Sodium hyaluronate (200 mg) was dissolved in 0.22 μm filter filtered water (10 ml) and Amberlite H^+ was added to attain a pH ~3. The mixture was stirred at room temperature for 1 hr. The mixture was filtered and the filtrate was dried by lyophilization. HA was collected as a white solid. HA (76 mg) was dissolved in double distilled (dd) water (3 ml) by sonication, followed by the dropwise addition of acetonitrile (2 ml). NMM (22 μl) was added and the solution was cooled to 4^{o}C in an ice bath. CDMT (18 mg) was added and the mixture was stirred at room temperature for 1.5 hr. An aqueous dispersion of SPION (30 mg) was added and the mixture was stirred at room temperature for 36 hours. The pH of the reaction mixture was brought to ~7 using Amberlite H^+, and the resin was then removed by filtration. The filtrate was purified by ultrafiltration (MWCO 100,000), and HA-SPION was collected. To prepare FITC-labeled HA-SPION, FITC (1.6 mg) dissolved in DMSO (1 ml) was mixed with HA-SPION (4 mg/ml, 10 ml), and the mixture was stirred in the dark at room temperature for 48 hours. The reaction mixture was diluted by water and purified by dialysis (MWCO 14,000) and ulrafiltration (MWCO 100,000). The percentage of FITC loading (w/w) was quantified by UV-vis spectroscopy.

3. Assessing DOX release in vitro

Equal amounts of lyophilized DOX-HA-SPION (2.5 mg) were suspended in PBS (pH 7.4), aqueous HCl solution (pH 5.0), and aqueous NaOH solution (pH 9.0) respectively to a final volume of 1.25 ml. At specific time points, the tubes were centrifuged. 100 µl samples of the supernatant were drawn from each tube and transferred to a 96 well black plate (clear bottom). The release of DOX was assessed by measuring fluorescence on a plate reader (excitation wavelength 483 nm, emission wavelength 580 nm). When the measurement was done, the samples were returned to their respective tubes. After 24 hr, the pH of the tube with pH 7.4 was adjusted to 4.0, while pH of the tube with pH 5.0 was adjusted to 4.5 by adding 1M HCl solution. The continual release of DOX was assessed by fluorescence measurement as described above. To determine the total amount of DOX in the sample, 6N HCl (1.3 ml) was added to the nanoparticles to hydrolyze the core and the amount of DOX that was not released was determined by measuring fluorescence of the resulting solution. The fluorescence of unreleased DOX was added to the highest measured released DOX. The percentage of DOX released at a given time point is:

% DOX released = [(Fluorescence of released DOX)/Total fluorescence] x 100]

To study the stability of HA backbone under acidic conditions, FITC-ADH-HA conjugate[32] (1 mg) was dissolved in aqueous HCl solution (pH 4.5, 1 mL), and the solution was transferred to a centrifugal filter tube (Millipore, MWCO 10 kDa). At specific time points, the solution was centrifuged and the fluorescence of the filtrate (100 µl aliquots) was determined on a plate reader (excitation wavelength 488 nm, emission wavelength 520 nm). The retained FITC-ADH-HA conjugate was re-dissolved in aqueous HCl solution (pH 4.5, 1 mL). The percentage of FITC released at a given time point is:

% FITC released= [(Fluorescence of FITC in filtrate/fluorescence of initial solution) x 100

4. Competitive ELISA assay

IgG-Fc (100 μL) was added to the wells of a 96-well microtiter plate, and the plate was stored at 4^{o}C overnight. Unbound IgG-Fc was removed and the wells were washed with 0.5% PBS-Tween 20 (PBST, 200 μL) three times. After the final wash, the residual liquid in the wells was removed by tapping the plate on soft paper tissue. The wells were blocked by adding 5% BSA in PBS (200 μL), and the plate was incubated at 37^{o}C for 2 hr. The wells were washed as above. CD44-Fc γ chimera (0.2 μg/well, 100 μL) in PBS was added to the wells, and the plate was incubated at 37^{o}C for 45 min. The wells were then washed as described above using 0.05% PBST. Biotinylated HA (b-HA) (0.5 μg/well, 100 μl), b-HA+HA (31 kDa) (2.5 μg/well), b-HA+HA-SPION, or b-HA+SPION were added according to a predetermined design. The HA-SPION concentrations used to generate IC_{50} curve (Figure S8) were 73.2, 18.3, 4.58, 1.14, 0.29, 7.14 x 10^{-2}, 1.78 x 10^{-2}, and 4.4 x 10^{-3} μg-HA/mL and b-HA concentration was 1.5 μg/well. The plate was stored at room temperature for 2 hr. The wells were then washed as described above using 0.05% PBST. Avidin-HRP (1:2000 dilution, 100 μL) in 0.2% BSA-PBS was added to each well and the plate was stored at room temperature for 1 hr. The wells were washed as described above using 0.05% PBST and twice with PBS buffer. A fresh TMB solution was prepared by dissolving TMB (5 mg) in DMSO (2 ml), followed by citrate phosphate buffer (18 mL). 30% H_2O_2 (20 μL) was added just before use. TMB solution (100 μL) was added to the wells, and the plate was stored at room temperature in the dark for 15 min, or until the blue color appears. The reaction was then quenched by adding 0.5 M H_2SO_4 (50 μL) to each well with positive wells turning yellow. Optical absorbance was directly measured through the bottom of the microtiter plate using an automated plate reader (Bio-Rad) at 450 nm.

5. High-Resolution Magic Angle Spinning (HRMAS) NMR

HRMAS NMR experiments were carried out on a Varian Inova- 500 NMR spectrometer equipped with a 4 mm gHXNanoprobe (Variannmr Inc., Palo Alto, CA) available at the University of Tennessee Health Science Center (Memphis, TN). The HR-MAS probe with internal lock is capable of performing either direct or indirect (inverse) detection experiments. Magic angle spinning (MAS) experiments were performed at spinning rates of up to 2.5 kHz using a 40 μL glass rotor. HA-SPION, ADH-HA-SPION, or DOX-HA-SPION were dissolved in D_2O solvent and were further diluted at different concentrations with D_2O to find out the concentration limit to the NMR signal broadening. HRMAS ^1H NMR spectra were obtained using 100-600 scans for each experiment. The sample temperature was regulated with an accuracy of ± 0.1 °C.

6. Determining the r_2 values for the various SPIONs*

Five different dilutions of the various SPION were prepared to a final volume of 5 mL in 15 mL-centrifuge tubes (Corning). The tubes were placed on a polystyrene tube holder. All MRI experiments were carried out on a GE 3T Signa® HDx MR scanner (GE Healthcare, Waukesha, WI). To evaluate the r_2* characteristics of the nanoparticles, the following parameters were used: head coil, 3D fast spoiled gradient recalled echo sequence, flip angle = 15°, 16 echo times (TEs) = 2.1 ms, 4.6 ms, 7.0 ms, 9.4 ms, 11.8 ms, 14.3 ms, 16.7 ms, 19.1 ms, 21.5 ms, 24.0 ms, 26.4 ms, 28.8 ms, 31.2 ms, 33.7 ms, 36.1 ms, and 38.5 ms, time of repetition (TR) = 41.9 ms, receiver bandwidth (rBW) = ± 62.5 kHz, field of view (FOV) = 16 cm, slice thickness = 1.5 mm, number of slices = 16, acquisition matrix = 256 × 256, number of excitation

(NEX) = 1, and scan time = 1 min 55 sec. [Fe] was plotted against (1/T2*), and r_2* is the slope of the generated straight line.

7. CD44 expression on SKOV-3 cells

7.1. By laser confocal microscopy: Cells (2 x 10^5 cells/well) were inoculated in two wells of a 4-well borosilicate plate and incubated for 24 hrs at 37^{o}C and 5% CO_2. The cells were then fixed using 10% neutral buffered formalin (0.75 mL) for 15-20 min. The cells were washed twice (10 min each) with DPBS containing 0.05% Tween 20 (washing buffer) on an orbital shaker. To one well, PE-Cy^{TM}5 Rat Anti-Mouse CD44 (25 µg/ml-1%BSA/DPBS, 200 µL) was added while the other well was used as control where 1% BSA-DPBS (200 µL) was added. The plate was incubated for 3 hours at 4^{o}C. After incubation, the wells were washed twice with the washing buffer for 10 min as described above. The wells were then washed with dd water for 5 min to avoid PBS crystallization on the sample. Images were collected on an Olympus FluoView 1000 LSM confocal microscope.

7.2. By Western blotting: The lysis buffer contained the following for 10 mL culture plates: 500 µL RIPA buffer (Sigma) + 20 µL Protease inhibitor cocktail + 2.5 µL Phenyl methyl sulfonyl chloride (PMSF, phosphatase inhibitor). The cell culture medium was removed using an aspirator and the cells were washed with ice cold cell culture grade PBS twice. The lysis buffer was added and the plate was rocked in a cold room till cell debris starts to float around (approximately 10-15 min). The plate surface was scraped using a cell scraper, and the contents of the plate were transferred to precooled eppendorf tubes, followed by sonication in ice containing sonicator for 10 sec. The samples were centrifuged (14000 rpm) for 5 min at 4°C, and the supernatant was collected. A Bradford assay was conducted to determine the total protein

concentration in the cell lysate. 20 μg of protein together with high-molecular weight prestained standards were loaded on a non reducing-type gel (Stacking gel: 18%; resolving gel: 10%). The gel was subjected to electrophoresis (200 V), and the blots were then transferred to PVDF membrane (60 V). After blocking the membrane using 4% non-fat milk in TBS-tween (TBST) buffer, the membrane was first treated with mouse anti-human CD44 antibody (primary, 156-3C11, Cell signaling) diluted to 1:1000 in 4% non-fat milk in TBST at 4°C overnight followed by goat anti-mouse HRP antibody (secondary, Biorad) for 1 h. The membrane was sprayed with a chemiluminescent HRP detection reagent (Denville Scientific) and developed onto autoradiography film. To perform β-actin detection (positive control), the same membrane was incubated with the mouse antihuman β-actin peroxidase antibody AC-15 (Sigma-Aldrich,) diluted at 1:40,000 in 4% milk/TBST for 1 h at room temperature. The blot was developed as described before.

8. Prussian blue staining

SKOV-3 cancer cells (5 x 10^5 cells/well) were allowed to attach overnight in a 12-well plate at 37^{o}C and 5% CO_2. The media was removed and the cells were washed with PBS. SPION and HA-SPION (48 μg/ml-Fe, 1 ml) were added and the cells were incubated for 18 hr after which the supernatant was removed and the cells were washed three times with PBS. The cells were fixed with 10% neutral formalin (0.5 ml/well) for 5 min, and then washed with PBS twice. To each well was added 0.5 ml of a 1:1 mixture of 4% potassium ferrocyanide (II) trihydrate and 4% HCl solution (in PBS) and the cells were incubated in the dark at 37^{o}C for 30 min. The staining solution was removed, and the cells were washed with PBS and counterstained with nuclear fast red (0.3 mL/well) for 3 min. The supernatant was removed and

the cells were washed with water. Images were taken on an Olympus CKX41inverted light microscope.

9. Determination of cellular uptake by flow cytometry

Cells (2 x 10^5 cells/well) were allowed to attach in a 24-well plate overnight at 37^{o}C and 5% CO_2. The cells were then washed twice with PBS. 1 ml of various concentrations of FITC-SPION and the equivalent FITC-HA-SPION were added to different wells and the plate was incubated for 18 hr at 37^{o}C and 5% CO_2. The cells were then washed with DPBS (3 times) and trypsinized with 0.25% trypsin-EDTA (1 mL). Trypsin was neutralized with serum-containing DMEM (5 times), and the cells were collected by centrifugation (500 g). The cells were resuspended in serum-containing DMEM (300 µL) and transferred to FACS tubes. The cells were stored on ice till the time of FACS analysis. Propidium iodide (PI) (100 µg/ml, 3.3 µL) was added at the time of analysis.

10. Blocking the CD44 mediated uptake of HA-SPION

SKOV-3 cancer cells (2 x 10^5 cells/well) were allowed to attach in a 24-well plate overnight at 37^{o}C and 5% CO_2. The supernatant was removed, and the cells were washed twice with PBS. Anti-CD44 monoclonal antibody, clone MEM 85 (150 µL), was diluted in 1% BSA/PBS (2.85 mL). Some wells received the MEM-85 antibody solution (25 µL mAb/well, 500 µl), while the control wells received 1% BSA-PBS. The cells were incubated at 37^{o}C for 1 hr. The cells were then washed twice with PBS. FITC-HA-SPION (70 µg/mL, 1 mL) and FITC-SPION (25 µg/mL, 1 mL) were added. The plate was incubated at 37^{o}C for 1 hr after which the cells were washed with PBS (3 times) and trypsinized (500 µl trypsin/well). The cells were collected and trypsin was neutralized with serum-containing DMEM medium. After four

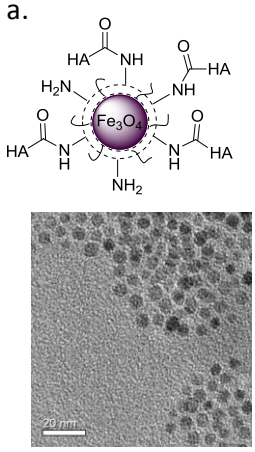
washes, the cells were pelleted (500 g, 4°C, 5 min). The supernatant was removed with the cells resuspended in serum-containing DMEM medium (300 μL) and transferred to FACS tubes. The cells were stored on ice till the time of the FACS analysis. PI (100 μg/ml, 3.3 μL) was added right before the measurements.

11. MR evaluation of uptake of HA-SPION and Feridex by SKOV-3 ovarian cancer cells

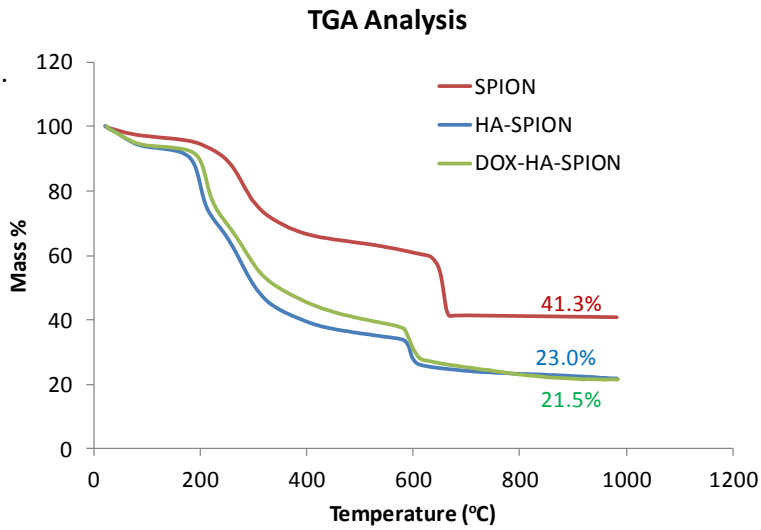
SKOV-3 cells (1 x 10^6 cells/well) were cultured in a 6-well plate for 12 hrs at 37°C and 5% CO_2. The culture media was removed followed by washing the cells with PBS (2 times). HA-SPION or Feridex in serum-free DMEM medium ([Fe] = 0.32 mg-Fe/mL, 2 mL) was added to two wells each. The cells were incubated at 37°C for 18 hr. The media was then removed and the cells were washed with PBS (3 times). The cells were trypsinized, and collected by centrifugation. Cells that received the same nanoparticle were combined in one Eppendorf tube (total of 2 x 10^6 cells/tube) and the cells were washed in PBS and centrifuged (2 times). After removing the supernatant, the cells were re-suspended in 1% agarose by vortexing. The agarose was allowed to solidify at room temperature with the samples stored at 4°C till MRI scanning. To evaluate the T2* effect of cellular uptake of the nanoparticles, the following parameters were used: wrist coil, 3D fast spoiled gradient recalled echo sequence, flip angle = 15°, 16 TEs = 2.5 ms, 5.1 ms, 7.7 ms, 10.2 ms, 12.8 ms, 15.3 ms, 17.9 ms, 20.5 ms, 23.0 ms, 25.6 ms, 28.1 ms, 30.7 ms, 33.3 ms, 35.8 ms, 38.4 ms and 40.9 ms, TR = 41.9 ms, rBW = ± 62.5 kHz, FOV = 16 cm, slice thickness = 1.5 mm, number of slices = 16, acquisition matrix = 256 × 256, NEX = 1, and scan time = 3 min 49 sec.

Figures in supporting information

a.



b.



c)

Sample	Hydrodynamic diameter		ζ- potential (mV)	% Weight loss (TGA)
	Z-average size (nm)	Polydispersity index (PDI)		
SPION	61	0.220	+26 ± 6.95	58.7
HA-SPION	114	0.151	-47 ± 7.75	77.0
DOX-HA-SPION	122	0.227	-44.5 ± 5.56	78.5

The hydrodynamic diameters of the NPs were larger than the diameters shown by TEM. This is due to the fact that TEM measures the size of the NP core under vaccum, while the hydrodynamic diameter is a measure of the size of the core and the total hydrated coating in solution.

All the NPs are quite homogenous as evident from the small PDI numbers. Upon HA functionalization of SPION, the zeta potential of the NPs changed from +26 (SPION) to -47 (HA-SPION) mV. This is because SPION has free amine groups on the surface, which should be protonated and hence positive charged at neutral pH. Following HA immobilization, the positive charged amines were converted to the neutral amides. Coupled with the fact that HA is negatively charged, this rendered HA-SPION bearing netative zeta potential.

HA loading calculation based on TGA data

The SPION has 41.3% by weight the inorganic core and 58.7% the organic coating from the TGA weight loss. Upon HA immobilization, the weight of the total organic coating increased to 77.0%. Solving equation (0.587+x)/(0.587+0.413+x) = 0.770 gave x a value of 0.795. Thus, the weight of HA on HA-SPION was 0.770/(0.587+0.413+0.770)*100% = 44%.

d) HRMAS-NMR spectra showed the characteristic NMR signals from ADH and DOX upon functionalization of HA-SPION.

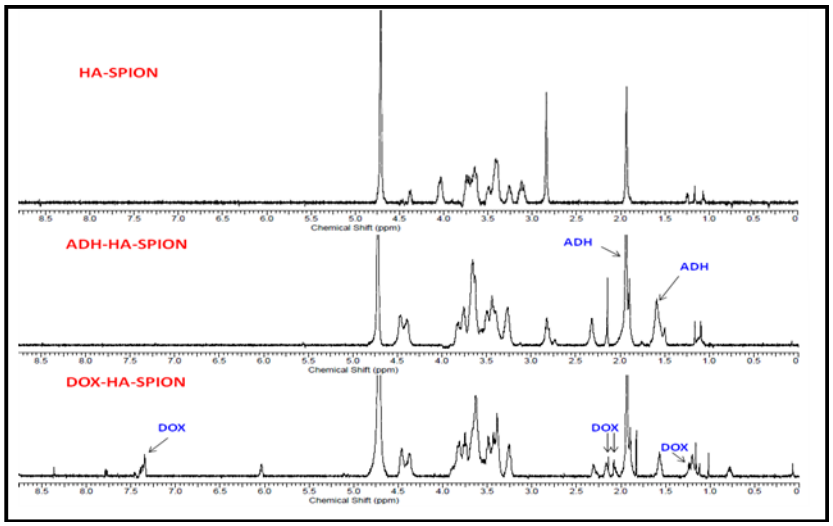


Fig. S1. Characterization of various HA-coated iron oxide nanoparticles. a) TEM of HA-SPION (the scale bar is 20 nm); b) TGA of SPION, HA-SPION, and ADH-HA-SPION; c) Table summarizing the DLS, polydispersity indices (PDI), zeta potential, and TGA data for SPION, HA-SPION, and DOX-HA-SPION; d) HRMAS-NMR for HA-SPION, ADH-HA-SPION and DOX-HA-SPION.

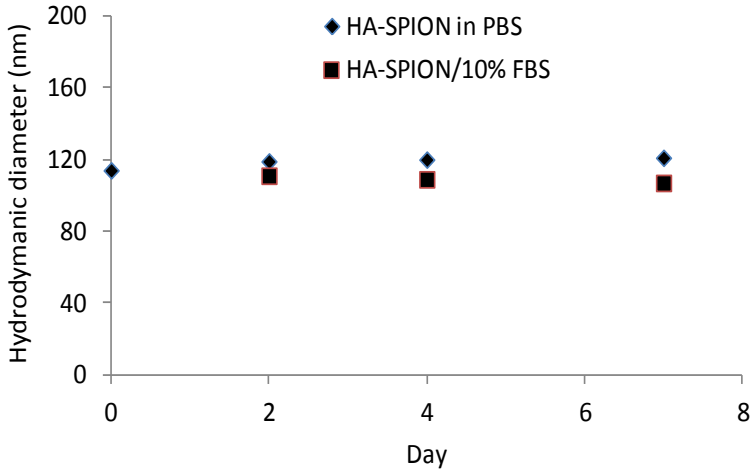


Fig. S2. Hydrodynamic diameters of HA-SPION upon incubation with PBS or 10% FBS containing media. The insignificant changes in its hydrodynamic sizes showed that the HA-SPION was colloidal stable in PBS buffer and in serum containing media. The serum proteins did not induce aggregation of the NPs.

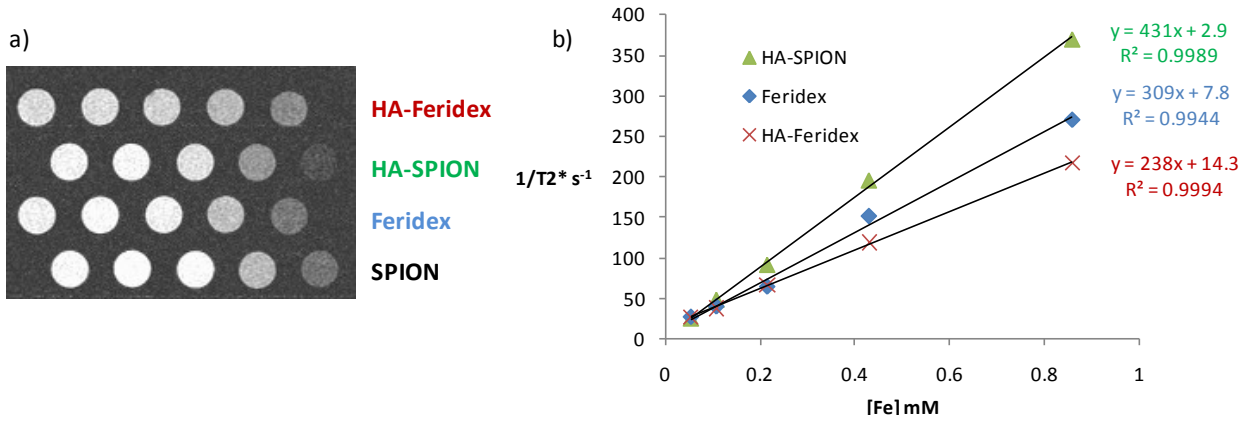


Fig. S3. a) MR images of the four types of magnetic nanoparticles with increasing concentrations. The amounts of iron for each column are the same. b) T2* relaxation rate (r_2*) of magnetic nanoparticles vs. iron concentration. The relaxivity of SPION is very similar to that of Feridex (data for SPION not shown).

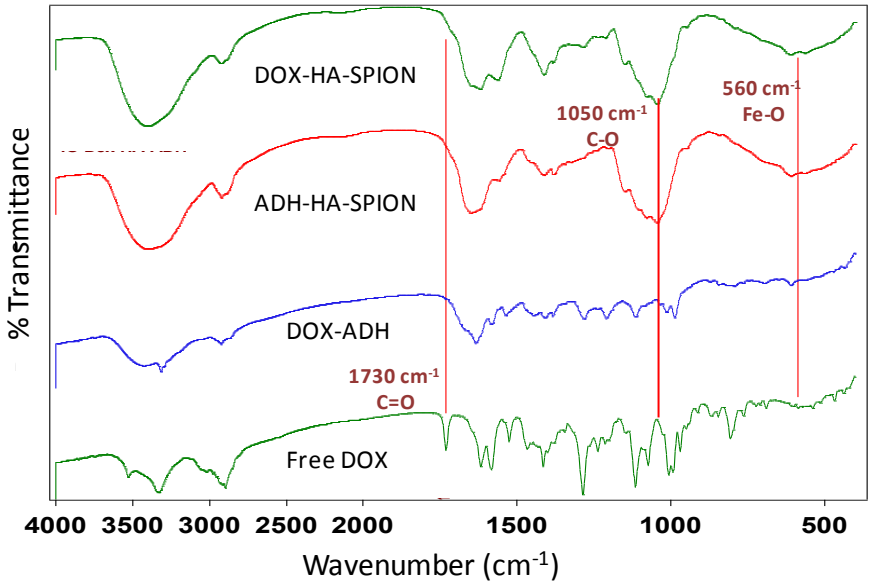


Fig. S4. Comparison of IR spectra of free DOX, DOX-ADH adduct, ADH-HA-SPION and DOX-HA-SPION confirmed the immobilization of DOX on nanoparticle surface.

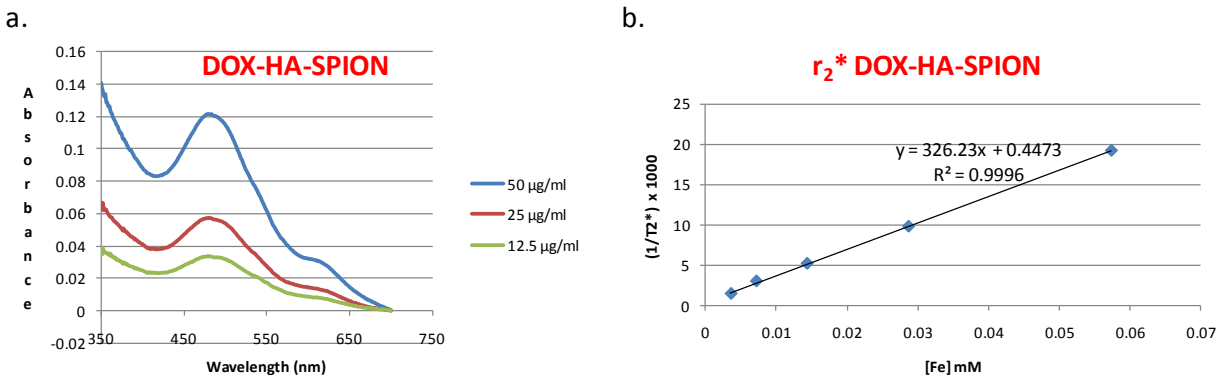


Fig. S5. A) UV-vis spectra of DOX-HA-SPION; b) T2* relaxation rate (r_2*) of DOX-HA-SPION vs. iron concentration.

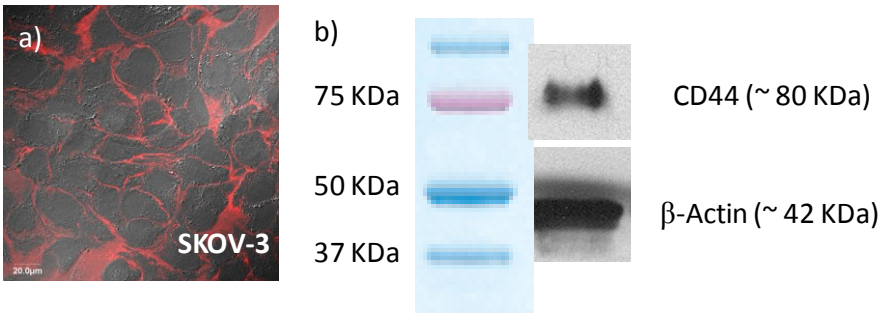


Fig. S6. a) Overlay of confocal microscopy images of PE-Cy5 channel with laser images of SKOV-3 cells after the cells were stained by PE-Cy5 labeled anti-CD44 mAb IM7. b) Western blot confirmed the expression of CD44 in SKOV-3 cells.

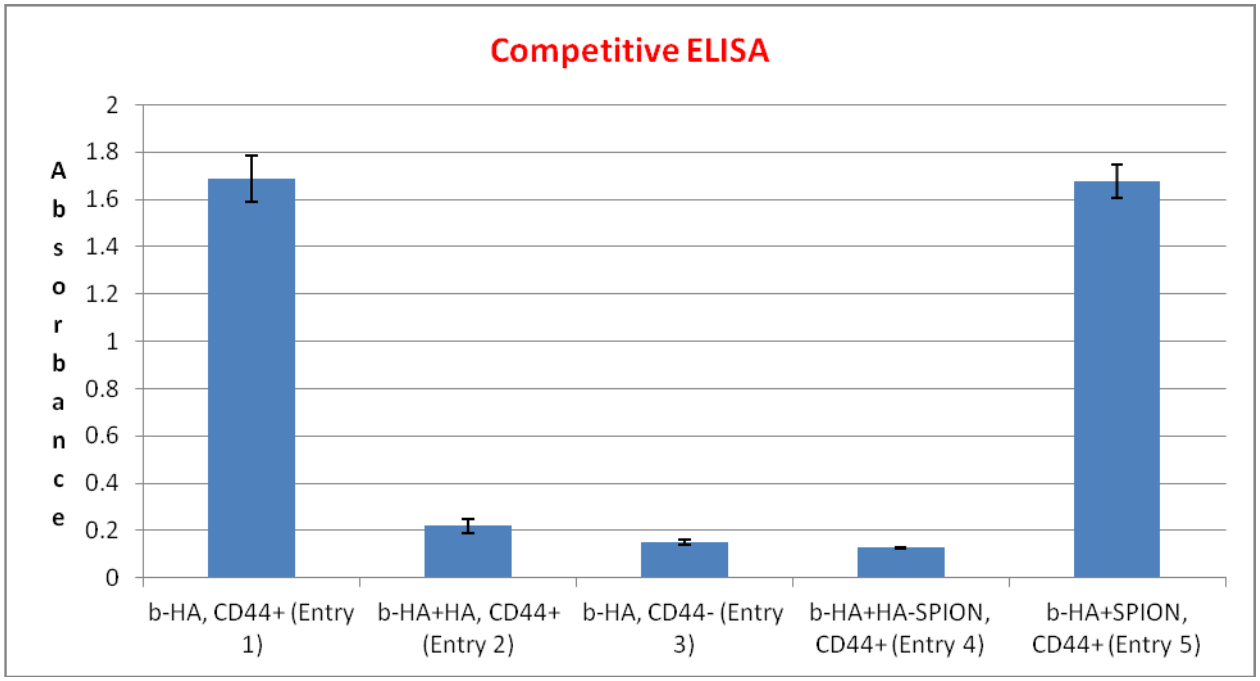


Fig. S7. Competitive ELISA data collected when SPION and HA-SPION competed with biotinylated-HA (b-HA) for CD44 binding. After being coated with IgG-Fc, the wells were blocked with BSA. Some of the wells received CD44-Fc γ and/or b-HA according to a pre-designed setup. Avidin-HRP was then added followed by 3,3',5,5'-tetramethoxybenzidine (TMB) to generate a blue color that was quenched by H_2SO_4. The intensity of the resulting yellow color is proportional to the amount of b-HA bound to CD44. As expected, the highest optical absorbance was obtained when b-HA (0.5 μg/well) and CD44 were added in one well (entry 1). However, when HA (31K, 2.5 μg/well) polymer was added with b-HA, the absorbance dropped dramatically (entry 2) indicating that HA polymer competed with b-HA for binding to CD44. Minimal absorbance was observed when b-HA was added to the wells that did not receive CD44 (entry 3). The binding of b-HA to CD44 was inhibited when HA-SPION (1 μg/mL) was added (entry 4). This result confirmed that HA retained it binding ability to CD44 after being linked to the surface of SPION. On the other hand, SPION (100 μg/mL) did not compete with b-HA for CD44 as a maximal absorbance was observed (entry 5). This result confirmed that the presence of HA is indispensible for the binding of the nanoparticles to CD44.

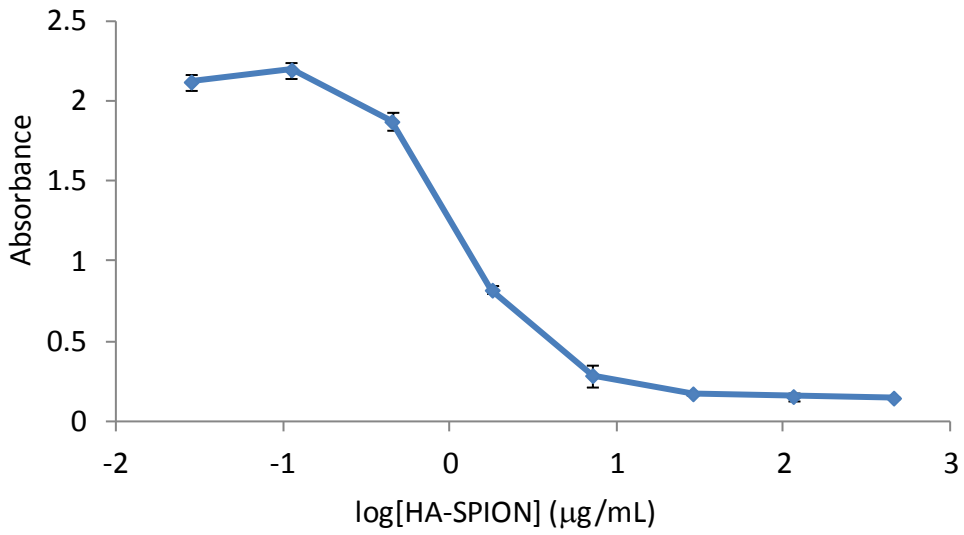


Fig. S8. HA-SPION competitively inhibits the binding between CD44 immobilized on a microtiter plate and a HA polymer with IC_{50} = 1.3 μg/mL.

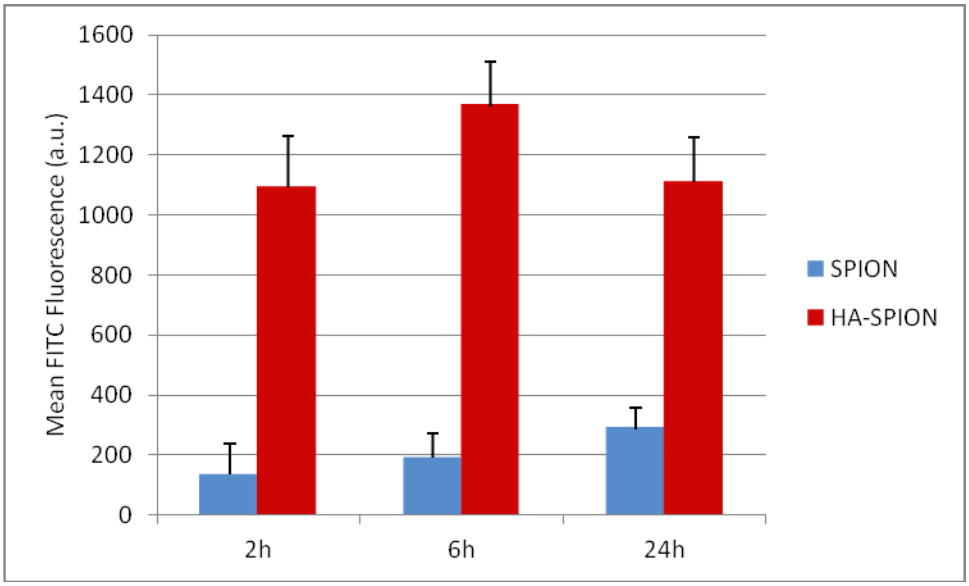


Fig. S9. Time dependent cellular uptake of SPION and HA-SPION by SKOV-3 ovarian cancer cells.

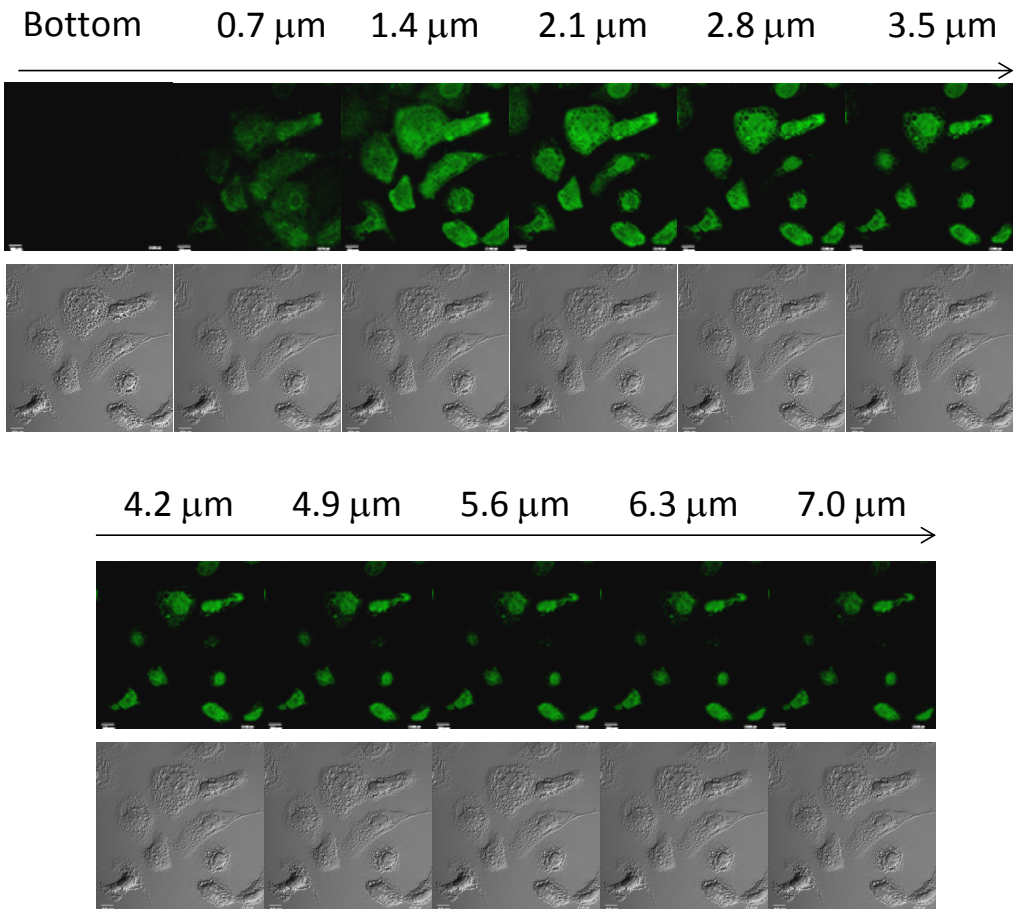


Fig. S10. Confocal microscopy Z section of SKOV-3 cells upon incubation with FITC-HA-SPION and washing off the unbound particles. The top row was the FITC channel starting from the bottom of the cell toward the top at 0.7 μm for each section. The bottom row was the corresponding DIC images. The scale bar is 10 μm.

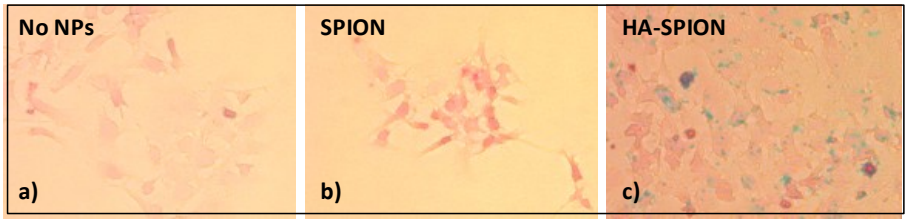


Fig. S11. Prussian blue staining of a) SKOV-3 cells; b) SKOV-3 cells after incubating with SPION for 5 hours and washing off the unbound particles; and c) SKOV-3 cells after incubating with HA-SPION for 5 hours and washing off the unbound particles.

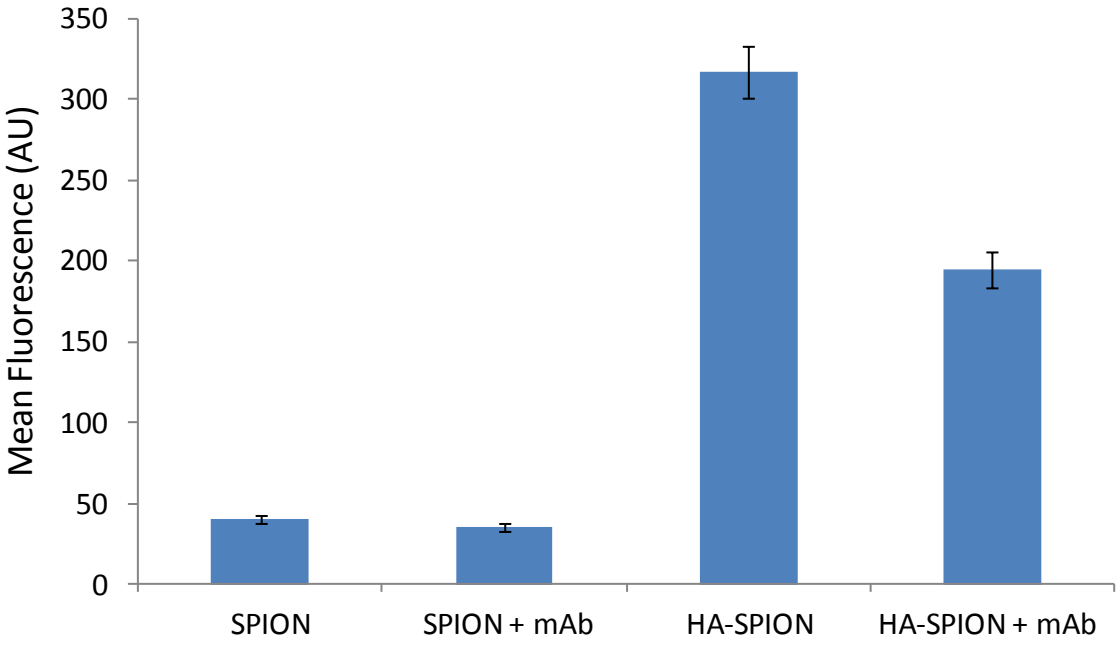


Fig. S12. Cellular uptake of SPION and HA-SPION by SKOV-3 cells as measured by flow cytometry in the absence and presence of partially blocking mAb MEM-85. The mAb significantly reduced the uptake of HA-SPION, but not SPION.

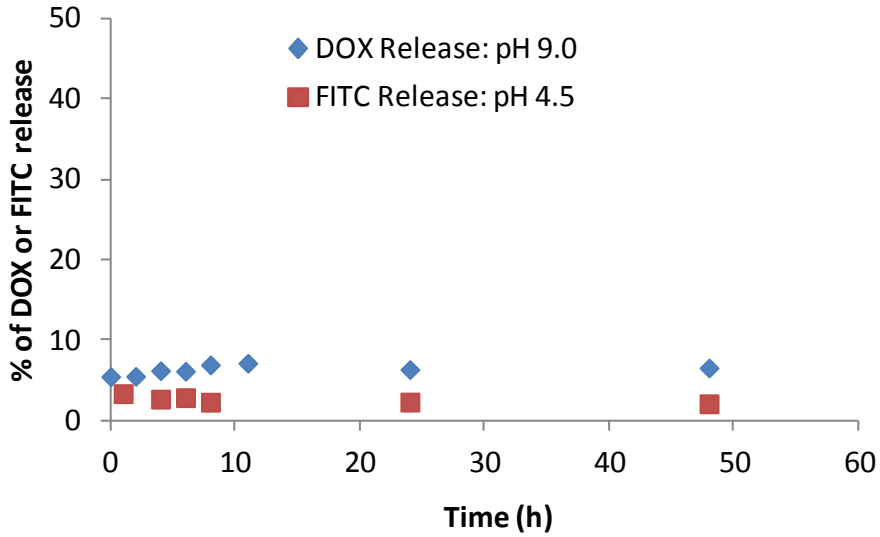


Fig. S13. Time course of DOX release from DOX-HA-SPION at pH 9.0 and FITC release from FITC-HA at pH 4.5. Minimum releases were observed under these conditions.

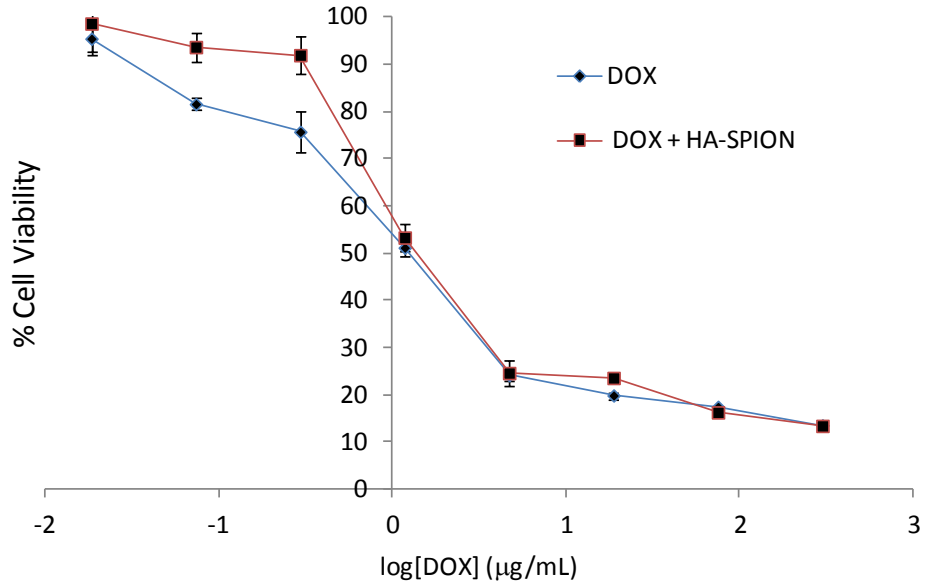


Fig. S14. MTS cell viability assay of SKOV-3 cells of free DOX in the presence or absence of HA-SPION. The HA-SPION did not significantly impact the toxicity of free DOX.

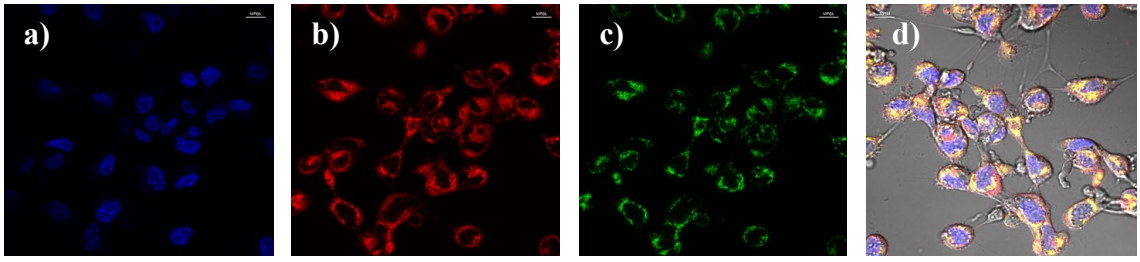


Fig. S15. Confocal microscopy images of live SKOV-3 cells after incubation with DOX-HA-SPION for 1 h. (a) Hoechst 33342 channel showing location of nucleus; (b) DOX channel showing location of the NPs; (c) Rhodamine 123 channel showing location of mitochondria; and (d) overlay of images in (a-c) with the laser image.