Promising tamoxifen-loaded biocompatible hybrid magnetic nanoplatforms against breast cancer cells: synthesis, characterization and biological evaluation

Diego Cadena Castro,[a,b] Gerardo Gatti,[c] Sandra E. Martín, [a,b] , Paula M. Uberman,*[a,b] and Mónica C. García *[d,e]

[a] Universidad Nacional de Córdoba. Facultad de Ciencias Químicas. Departamento de Química Orgánica. Córdoba. Argentina.

[b] Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET. Instituto de Investigaciones en Fisicoquímica de Córdoba, INFIQC. Córdoba. Argentina.

[c] Fundación para el Progreso de la Medicina. Laboratorio de Investigación en Cáncer. Córdoba, Argentina.

[d] Universidad Nacional de Córdoba. Facultad de Ciencias Químicas. Departamento de Ciencias Farmacéuticas. Córdoba. Argentina.

[e] Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET. Unidad de Investigación y Desarrollo en Tecnología Farmacéutica, UNITEFA. Córdoba. Argentina.

Corresponding Authors

*Paula M. Uberman: paula.uberman@unc.edu.ar. Address: Haya de la Torre y Medina Allende. Edificio Ciencias 2. Ciudad Universitaria X5000HUA, Córdoba, Argentina. Telephone/FAX: +54 351 5353867.

*Mónica C. García: mgarcia@unc.edu.ar. Address: Haya de la Torre y Medina Allende. Edificio Ciencias 2. Ciudad Universitaria X5000HUA, Córdoba, Argentina. Telephone/FAX: +54 351 5353865.

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1. General materials and methods

All materials were commercially available and used as received. All chemicals were reagent-grade and were used as received from the manufacturer. Iron (III) chloride ($FeCl_3$, Merck®); iron (II) chloride ($FeCl_2$, Stream Chemicals); ammonium hydroxide (NH_4OH), sodium hydroxide (NaOH); hydrochloric acid (HCl 37 % w/w); sodium chloride (NaCl); *L*-Cysteine hydrochloride (*L*-Cys, Parafarm®); *L*-Cystine (Parafarm®); trimethylsilyl chloride (ClTMS, Sigma Aldrich®), anhydride di-*tert*-butyl dicarbonate (Boc_2O, Sigma Aldrich®); triphenilphosphine (TPP, Sigma Aldrich®), sodium salt of hyaluronic acid (HA 97.4%) obtained from bacteria's fermentation (Parafarm®, MW = 1500 kDa, intrinsic viscosity = 23.6 $dL.g^{-1}$), 3-dimethylaminopropyl-N'-ethylcarbodiimida, (EDAC, Sigma Aldrich®), hydroxybenzotriazole (HOBt, Sigma Aldrich®), potassium bromide anhydrous (KBr, Sigma Aldrich®), 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent, Sigma Aldrich®), ethyl acetate (Sintorgan), glycerin pharmaceutical grade (Parafarm®), propylene glycol pharmaceutical grade (Parafarm®) and tamoxifen citrate (TMX, Parafarm®) were used as received.

All solvents were distilled before use: 1,4-dioxane (Cicarelli®), methanol (Cicarelli®), absolute ethanol (Cicarelli®).

^1H NMR was conducted on a high-resolution spectrometer Bruker Advance 400, in $CDCl_3$ or D_2O as solvent. TEM was conducted in a JEM-Jeol 1120 operating at 80 kV, at the IFFIVE Research Institute, INTA, Córdoba, Argentina. To characterize nanoparticles by TEM, samples were prepared depositing a drop of colloidal solution on a formvar-carbon coated cooper grid and dried at room temperature. The magnetic properties of the Fe_3O_4 were measured by employing a vibrating-sample magnetometer (VSM, Lake Shore 7300), the magnetization hysteresis loops were measured in fields between ±0.9 T, at room temperature, at the IFEG-CONICET-UNC-FaMAF, Córdoba.

FT-IR spectra were collected on an infrared microscope (Nicolet iN10, Thermo Scientific®, USA). Samples contained in KBr discs were scanned from 4000 to 400 cm^{-1} and the recording conditions were normal resolution; sample scan, 64/s. The spectra were recorded, processed and analyzed using the EZ OMNIC ESP 8.3.103 program. PXRD was performed using a PANalytical X'Pert Pro diffractometer (40 kV, 40 mA), in Bragg–Brentano reflection geometry with Cu Kα radiation

(λ=1.5418 Å). Data were gathered between 10° and 70° (2θ) in steps of 0.02 and a counting time of 24 s. The refinement of the crystal structure was performed by the Rietveld method using the FULLPROF program. TGA of samples was performed in a TGA Shimadzu Corporation Analytical model DTG–60, under air conditions. UV-vis determinations were performed using a SHIMATZU UV-Vis UV-1800 series.

Aqueous solutions or dispersions were prepared from analytical grade chemicals and Milli-Q water. All reactions were carried out under N_2 atmosphere, unless otherwise noted.

2. Synthesis of Fe_3O_4 nanoparticles as magnetic core

Magnetic core of magnetite (Fe_3O_4) nanoparticles was performed following the co-precipitation method [1], introducing some modifications to the general protocol. In a 100 mL three-neck round-bottom flask equipped with mechanical stirrer and N_2 inlet, 75 mL NH_4OH 0.7 M (molar ratio Fe:OH 3:22) were placed. In addition, a mixed solution of ferrous and ferric ions (molar ratio 1:2) was prepared by dissolving 2.36 mmol $FeCl_2$ in 6 mL of HCl solution (3 M) and 4.73 mmol $FeCl_3$ in 3 mL of decarbonated milli-Q water. The resulting solution of Fe^{2+}/Fe^{3+} was then quickly added to the NH_4OH solution under vigorous mechanical stirring (2000 rpm), and the suspension was continuously stirred under N_2 for about 30 min. The formation of Fe_3O_4 nanoparticles was immediately observed by the presence of a black precipitate. The magnetic precipitate was collected with the help of an external magnet of neodymium, and washed 4 times with 150 mL mixture of water:ethanol 1:1 to remove excess of reactants and by-products. Finally, the obtained Fe_3O_4 nanoparticles were redispersed in 75 mL ethanol and stored for further use. In addition, a part of them was separated and dried at room temperature under air for characterization.

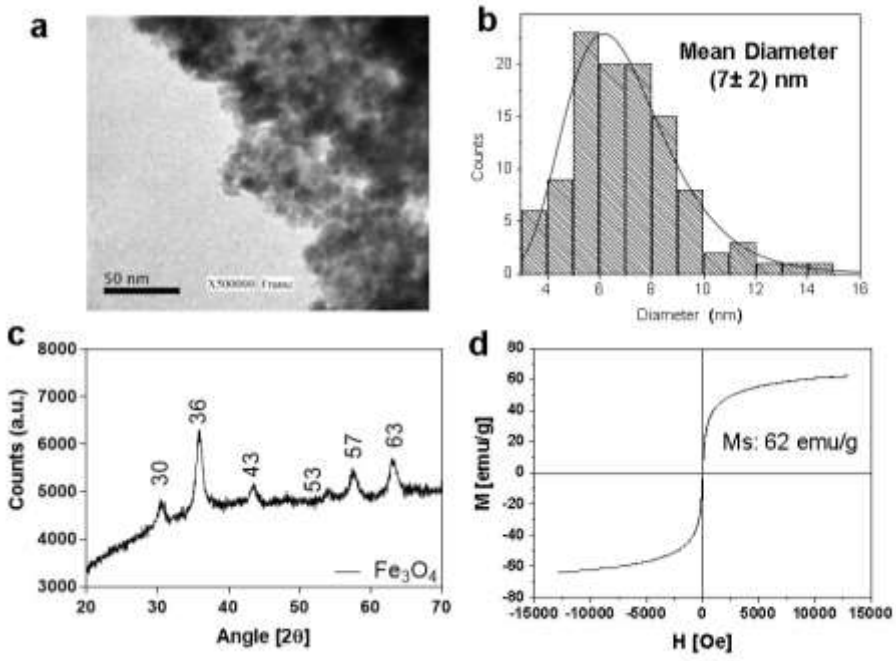

Figure S1. Characterization of Fe_3O_4 nanoparticles obtained by co-precipitation reaction by: a) TEM image, b) particle size distribution, c) PXRD, and d) VMS analysis.

3. Synthesis of Fe_3O_4 nanoparticles functionalized with *L*-Cysteine (Fe_3O_4-*L*-Cys)

Once the Fe_3O_4 nanoparticles were obtained, they were dispersed in 75 mL ethanol, and a 60 mM solution of *L*-Cys (in buffer carbonate pH= 8.8, 75 mL) was immediately added. The mixture was mechanically stirred (2000 rpm) and sonicated by 3 h. The magnetic powder was collected with an external magnet and washed 4 times with 150 mL mixture of water:ethanol 1:1. The as-obtained Fe_3O_4-*L*-Cys nanoparticles were dispersed in 110 mL milli-Q water for further functionalization with HA. A part of this conjugated was separated and dried at room temperature under air for characterization.

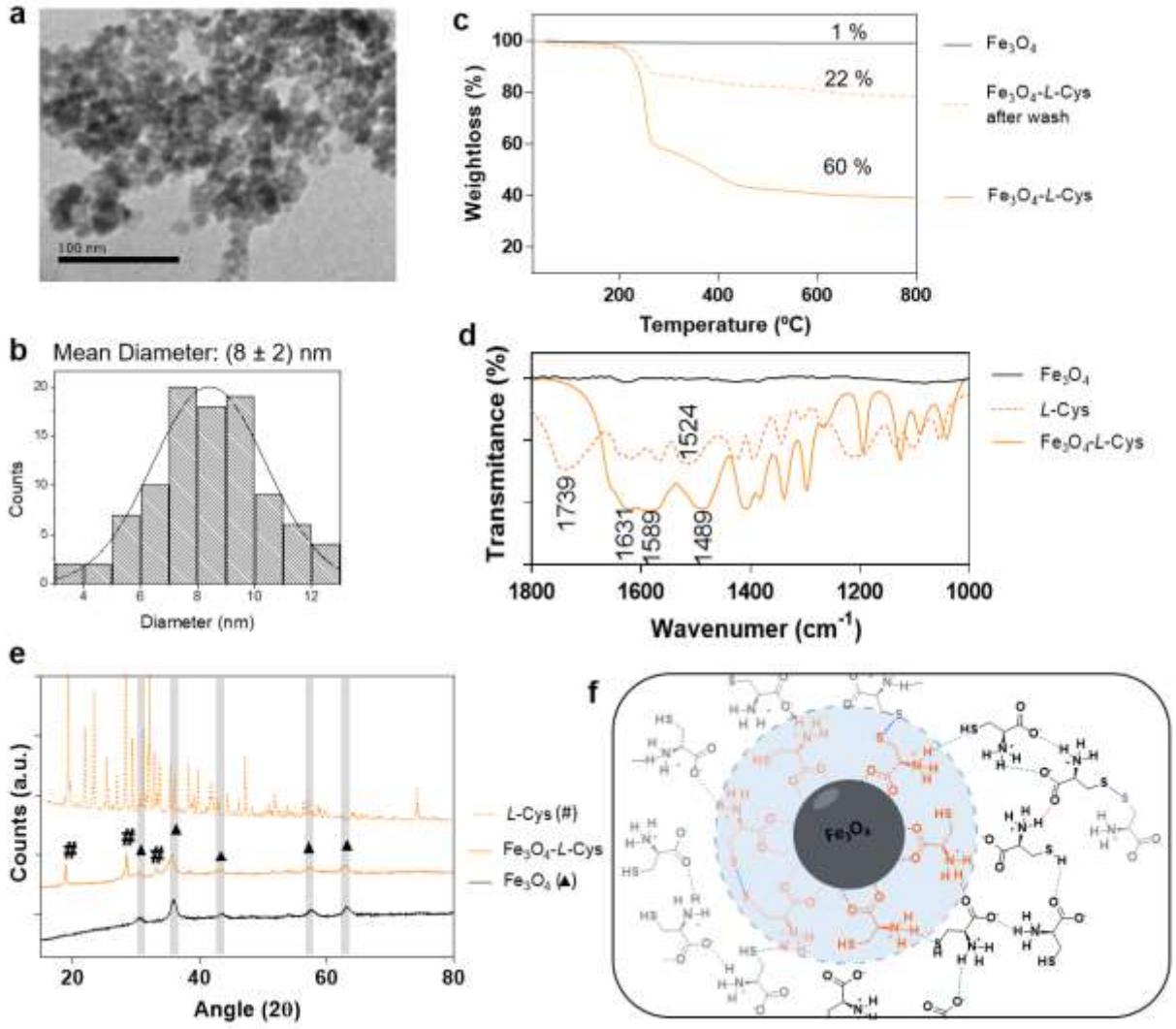

Figure S2. Characterization of Fe_3O_4-*L*-Cys conjugated by: a) TEM image and b) particle size distribution, c) TGA curves of Fe_3O_4, Fe_3O_4-*L*-Cys before and after exhaustive wash with water and d) FT-IR spectra of Fe_3O_4-*L*-Cys and its precursors (Fe_3O_4 nanoparticles and *L*-Cys). e) DRX diffractograms of bare Fe_3O_4 nanoparticles and conjugated with *L*-Cys. f) Schematic representation of the proposed adsorption for *L*-Cys onto Fe_3O_4 nanoparticles as a multilayer structure.

4. Characterization of Fe_3O_4 nanoparticles functionalized with *L*-Cysteine and hyaluronic acid (Fe_3O_4-*L*-Cys-HA HMNP)

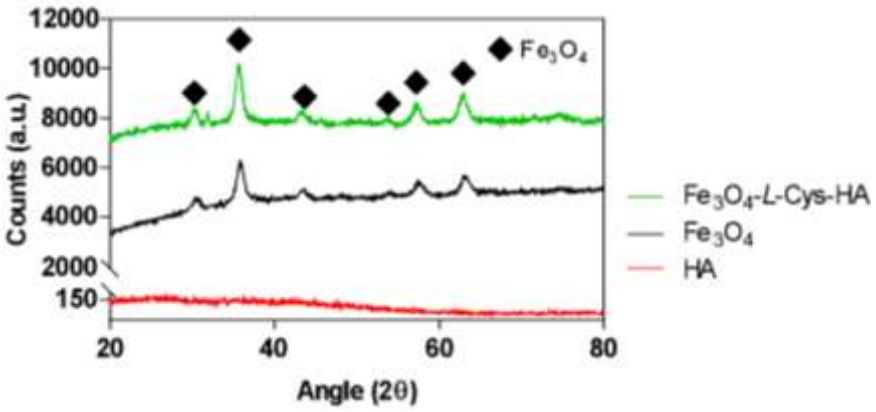

Figure S3: DRX diffractograms of bare Fe_3O_4 nanoparticles, HA and Fe_3O_4-*L*-Cys-HA HMNP.

5. Synthesis of hyaluronic acid modified with *L*-Cys (HA-*L*-Cys conjugate polymer)

In order to corroborate the incorporation of HA into the HMNP by formation of amide bond with NH_2 group of *L*-Cys, the *L*-Cys-HA polymer was separately synthesized [2].

Into a Schlenk tube dried, equipped with a magnetic stirrer and under N_2 atmosphere 0.100 g of AH dispersed into 25 mL of H_2O, were added. Next, under vigorous stirring 1.25 mmol EDAC and 1.25 mmol of HOBt, and the pH of the mixture was adjusted to 5.5 with HCl 0.1 M. The mixture was stirred at room temperature for 15 min. This procedure was performed to achieve the COOH group activation. After that, 1.25 mmol of *L*-Cys were added and the pH of mixture was readjusted until pH 5.5 with HCl 0.1M. The reaction was stirred at room temperature for 4 h. Then, the reaction mixture was dialyzed in tubing (molecular weight *cut-off* 12KDa) 2 times against NaCl 0.9 % p/v, for 6 h each time. With the aim to obtain a powder sample to work, the aqueous polymer solution was frozen and lyophilized at -50 ºC and 0.01 mbar (Rificor L-T8), stored for characterization and further use. The *L*-Cys-HA bioconjugate polymer was characterized by TGA, FT-IR and ^{1}H NMR analysis (**Figure S4**), and the obtained data were in agreement with those previously reported [2].

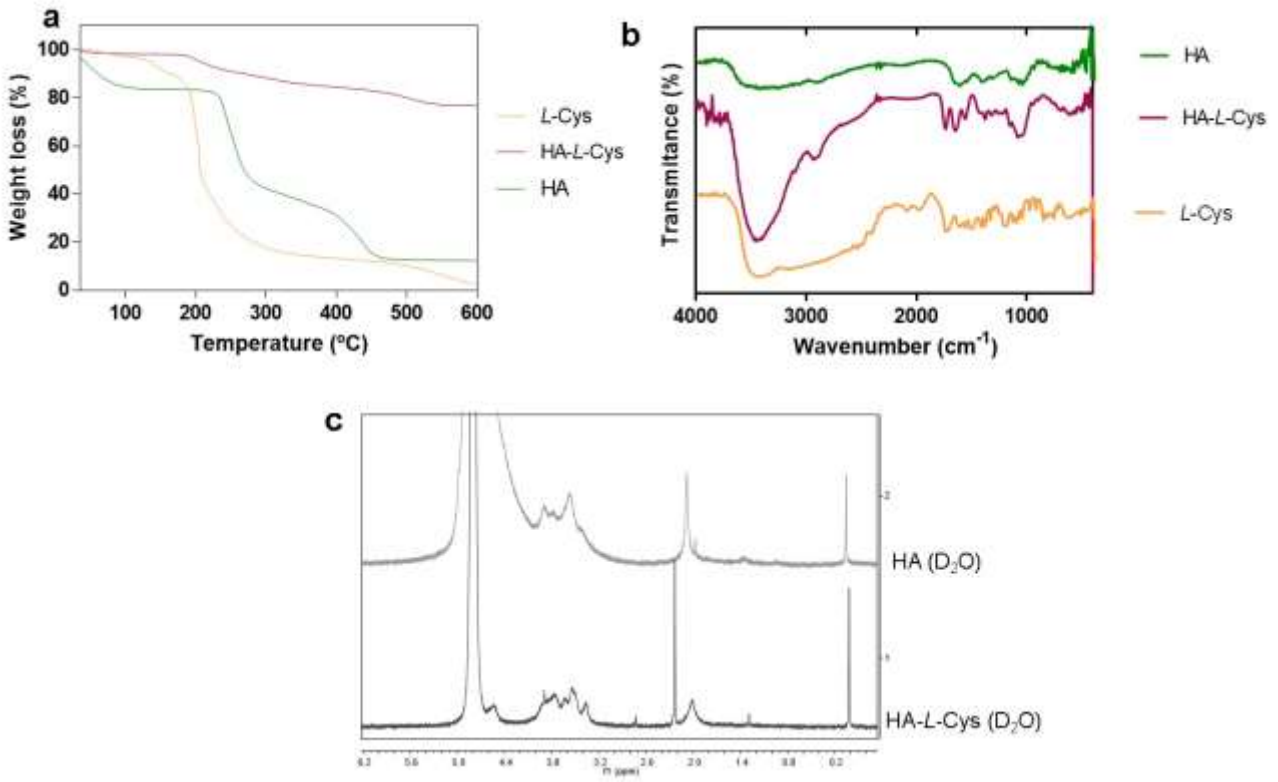

Figure S4: a) TGA curves and b) FT-IR spectra of the HA-*L*-Cys bioconjugate polymer and its precursors (hyaluronic acid (HA) and *L*-Cysteine (*L*-Cys)). c) ^{1}H NMR analysis of the bioconjugate and unmodified polymer HA.

6. Determination of thiol groups into HA-*L*-Cys conjugate polymer and Fe_3O_4-*L*-Cys-HA HMNP by Ellman's analysis

The amount of *L*-Cys incorporated into the HA polymer structure and HMNP was determined by Ellman's assay [3]. The amount of thiol group free was determined spectrophotometrically using 5,5'-dithiobis(2-nitrobenzoic acid) or Ellman's reagent as it was described previously [4], employing a standard curve obtained by solutions with increasing concentrations of *L*-Cys measuring at 412 nm. The molar extinction coefficient (ε) determined was 10,013 M^{-1} cm^{-1}. For each sample determination, a mother solution was prepared containing 4 mg of Ellman's reagent were dissolved into 1 mL of phosphate buffer pH 8.

Samples HA-*L*-Cys and Fe_3O_4-*L*-Cys-HA HMNP were treated by the following: 3 mg of sample were dissolved in 6 mL of phosphate buffer pH 8, and treated with 6 mL of a solution of $NaBH_4$ 10 mM under vigorous stirred for 30 min at 40 ºC. After that 6 mL of acetone and 1.8 mL of HCl 1 M were

added and the sample was stirred for 15 min. The samples were centrifuged and the supernatants were measured by UV-Visible spectrophotometer at 412 nm.

7. Characterization of Fe_3O_4 nanoparticles functionalized with HA (Fe_3O_4-HA HMNP)

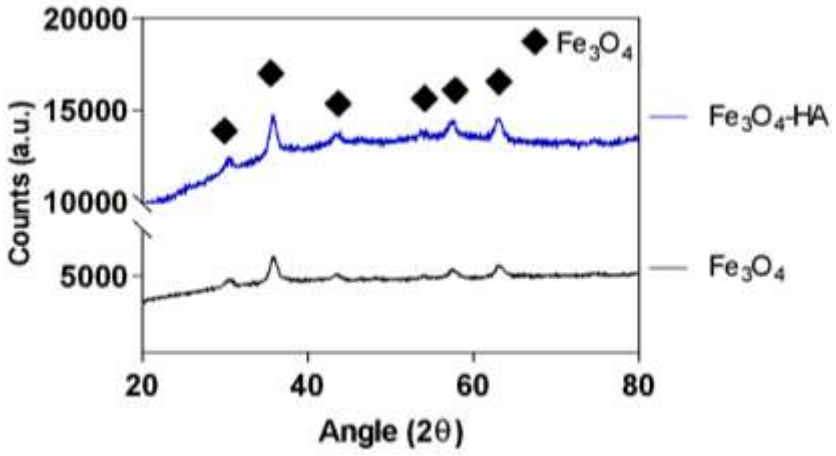

Figure S5: DRX diffractograms of bare Fe_3O_4 nanoparticles, HA and Fe_3O_4-HA HMNP.

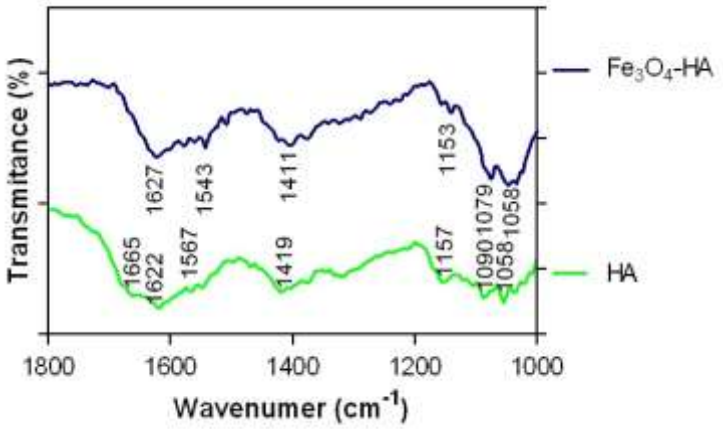

Figure S6: FT-IR spectra for HA and Fe_3O_4-HA HMNP.

8. *In vitro* cytotoxicity against normal breast cell lines

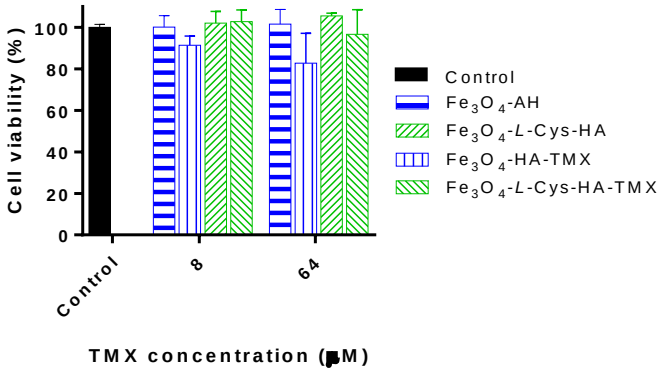

Figure S7: Cell viability percentage of HMNP and TMX-loaded HMNP with and without *L*-Cys, at two different concentrations on MCF-10A normal breast cell line. Non-significant differences were observed among the different groups.

9. References

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