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SPECIFIC TARGETING OF FOLATE RECEPTOR BY METHOTREXATE CONJUGATED MODIFIED MAGNETIC NANOPARTICLES: ENZYMATIC RELEASE AND CYTOTOXIC STUDY

Azam Sargazi, Keramat Kuhestani, Tayebe Nosrat Nahoki and Mostafa Heidari Majd*

Faculty of Pharmacy, Zabol University of Medical Sciences, Zabol, Iran

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Correspondence to Author:

Mostafa Heidari Majd

Faculty of Pharmacy, Zabol University of Medical Sciences, Zabol, Iran.

E-mail: mhmajd@zbu.ac.ir

ABSTRACT: Magnetic nanocarriers have been used for specific drug delivery to release therapeutic drugs into target cancer cells. We used bifunctional methotrexate (MTX) conjugated magnetic nanoparticles (MNPs) (~37 nm) engineered by dopamine-polyethylene glycol to targeted folate receptor (FR)-positive cancer cells. For this purpose, MTX was chemically loaded on to MNPs with *N*, *N*-dicyclohexylcarbodiimide (DCC) in the absence of NHS at 0°C. Activation process of MTX was analyzed via synthesis of different MTX methyl esters through HPLC instrument. The HPLC analysis showed that in a selective condition and in the presence of 1.1 equiv. of reactive agent, α -carboxyl group of MTX was more active. Enzymatic release study of MTX demonstrated that the highest rate of drug release was at pH=3.8 with protease enzyme (85.12%). Also, cytotoxicity assay revealed that Fe₃O₄-DPA-PEG-MTX NPs were able to target over expressed FR cell line (MCF-7) but did not have any effect on FR-negative A549 cells and significantly inhibit them.

INTRODUCTION: Targeted super paramagnetic nanoparticles have elicited great hopes for their potential and effective applications in tumor diagnostics, magnetic bio-separation, chemotherapy drug and gene delivery, hyperthermia managing tool, cell labeling, and contrast agent for magnetic resonance imaging (MRI) ¹⁻³. Although internalization of unmodified magnetic nanoparticles (MNPs) is limited by high surface energy and also short plasma half-life ⁴, surface-modified MNPs by biocompatible polymer can be used as an effective drug carrier in biological environment for controlled release of drug because of their tumor accumulation ability, suitable nano-scale size, and high hydrophilicity ⁵.

To prevent the plasma coating and elimination through reticuloendothelial system (RES), the MNPs surface is usually coated with stable, hydrophilic, and biocompatible polymers such as polyethylene glycol (PEG) ⁶⁻⁸. The PEGylated MNPs could increase the blood circulation time of particulate by their water-soluble ability and colloidal stability; also, they could endow an active functional group capable decorate surface of MNPs with other ligands such as FR targeting agents ^{5, 9, 10}.

As regards, FRs are over expressed on the cell membrane of several tumors ¹¹; conjugation of folic acid and its analogues such as methotrexate (MTX) onto the surface of MNPs provides not only both active and passive targeting capabilities but also delivery of an anticancer agent to tumor cells ^{1, 2, 12}. MTX, an antitumor drug having an effect on the DNA replication and cell proliferation, is used for the treatment of certain cancers such as leukemia, lymphoma, breast, head, and neck cancers ¹³⁻¹⁵. The clinical uses of MTX showed

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some disadvantages because of its very short plasma half-life. Therefore, cancer cells may show resistance to this drug in higher doses due to cellular efflux phenomenon; it can also induce dose-dependent side effects in normal cells such as bone marrow, gastrointestinal mucosa, hair, and renal system^{16, 17}. Thus, conjugation of MTX with some targeting carriers such as modified MNPs could increase the accumulation of drug in tumor cells and lead to reduction of side effects in normal cells and also decrease drug-resistance phenomena in target cells¹³.

In this study, we engineered MTX-conjugated PEGylated MNPs which could be utilized for simultaneous targeting and therapy of the FRs-over expressed tumor cells by an enzyme-dependent process.

MATERIALS AND METHODS:

Preparation of Fe₃O₄-DPA-PEG-NH₂ NPs:

The modified Fe₃O₄, Fe₃O₄-DPA-PEG-NH₂ NPs, was synthesized by a method reported in our previous manuscript^{2, 18}. Briefly, Iron(III) acetylacetonate [Fe(acac)₃](Merck, Hohenbrunn, Germany) (2.12 g, 6.0 mmol) was dissolved in a mixture of benzyl ether (Merck, Hohenbrunn, Germany) and oleylamine (Sigma-Aldrich, Steinheim, Germany) (ratio 30:30 mL) and was mixed by magnetic stirrer¹⁹⁻²¹. The solution was dehydrated at 120°C for 1 h using Dean–Stark apparatus under flow of argon, and then temperature was raised quickly to 270°C for 2 h under argon flow. The reaction mixture was cooled to room temperature (RT) and then 80 mL ethanol was added to the dark brown mixture and precipitated with centrifugation at 5000 rpm (Yield: 84.90%).

For the synthesis of DPA-PEG-NHBoc, three main steps were conducted as described previously^{2, 19}. In first step, polyethylene glycol(PEG)(Merck, Darmstadt, Germany) (10.0 g, 5.0 mmol), bromoacetyl chloride(Sigma-Aldrich, Steinheim, Germany) (1.75mL, 20.0mmol), and triethylamine (Merck, Darmstadt, Germany) (2.8 mL, 20mmol) were dissolved in 20mL dichloromethane. After one night, the light yellow Bis(2-bromoacetyl) polyethylene glycol(BBrAc-PEG) was precipitated in diethyl ether(Yield: 75.00%). In the second step,

BBrAc-PEG (7.50 g, 3.345 mmol) was dissolved in 250mL dichloromethane, and then dopamine hydrobromide (DPA) (Sigma-Aldrich, Steinheim, Germany) (0.817g, 3.487mmol), KI (0.277g, 1.6725mmol) and K₂CO₃ (1.615g, 11.707mmol) were added to the solution. The mixture was stirred overnight at room temperature under argon. Then, the (2-bromoacetyl) - (2'dopamineacetyl) polyethylene glycol (DPA-PEG-BBrAc) was precipitated by diethyl ether (Yield: 82.93%). In the third step, DPA-PEG-BrAC(6.00 g, 2.51mmol) and *N-tert*-butoxycarbonyl - 1, 2-ethylenediamine (NHBoc) (Alfa Aesar Company, Lancashire, UK)(0.475g, 3.012mmol) were dissolved in 250mL dichloromethane.

Then, KI (0.50g, 3.012mmol) and K₂CO₃ (1.757 g, 12.552 mmol) were added and stirred overnight at RT under argon flow. The (2-Boc-imino-ethylene-imino) - (2'-dopamineacetyl) polyethylene glycol (DPA-PEG-NHBoc) was precipitated with 30mL diethyl ether (Yield: 88.83%). For removing *N-tert*-butoxycarbonyl (Boc) residues from DPA-PEG-NHBoc, DPA-PEG-NHBoc(1.0 g, 0.04 mM) was dissolved in 20 mL dichloromethane. The solution was stirred by magnetic stirrer. Then, trifluoroacetic acid (1.5 mL) was added and stirred for 1 h at room temperature. Solvent was removed by rotary and product (DPA-PEG-NH₂) was washed (3×) with dichloromethane. The final brown color product was precipitated using diethyl ether.

Finally, Fe₃O₄ (0.5 g, 2.16 mmol) was dispersed in dichloromethane (50 mL). DPA-PEG-NH₂ (2.5 g) was added to the solution and stirred overnight under argon blanket at 25°C. After one night, solutions were sonicated for 15 min and then, Fe₃O₄-DPA-PEG-NH₂ was precipitated using hexane and gathered by centrifugation at 4000 rpm. The solid dark-brown products re-dispersed in 20mL ethanol. **Fig.1** (product 9) represents the overall procedure for the preparation of Fe₃O₄-DPA-PEG-NH₂.

Conjugation of methotrexate to Fe₃O₄-DPA-PEG-NH₂:

To obtain methotrexate (MTX) chemical conjugation, MTX (Sigma-Aldrich, Steinheim, Germany) (25 mg, 0.055 mmol) and *N*, *N*-

dicyclohexylcarbodiimide (DCC) (Alfa Aesar Company, Lancashire, UK) (13 mg, 0.066 mmol) were dissolved in 4 mL dimethyl formamide (DMF) (ratio 1:12)¹⁷. The reaction solution was stirred overnight at 0°C. The precipitate was removed by centrifugation at 5000 rpm (10 min, 4°C). Then, the modified Fe₃O₄-DPA-PEG-NH₂ (211.8mg, 0.066mmol of NH₂ groups) was dispersed in yellow supernatant and 10μL of triethylamine (0.072mmol) was added to the

reaction media. These two solutions were mixed overnight at room temperature. **Fig.1** (product 11) shows this step of the reaction. After one night, 40mL of diethyl ether was added to the yielded solution and the Fe₃O₄-DPA-PEG-MTX was collected using the magnetic bead separation system "Dynamag TM-50". Finally, Fe₃O₄-DPA-PEG-MTX washed with deionized water (3×) and re-collected with magnetic separation system.

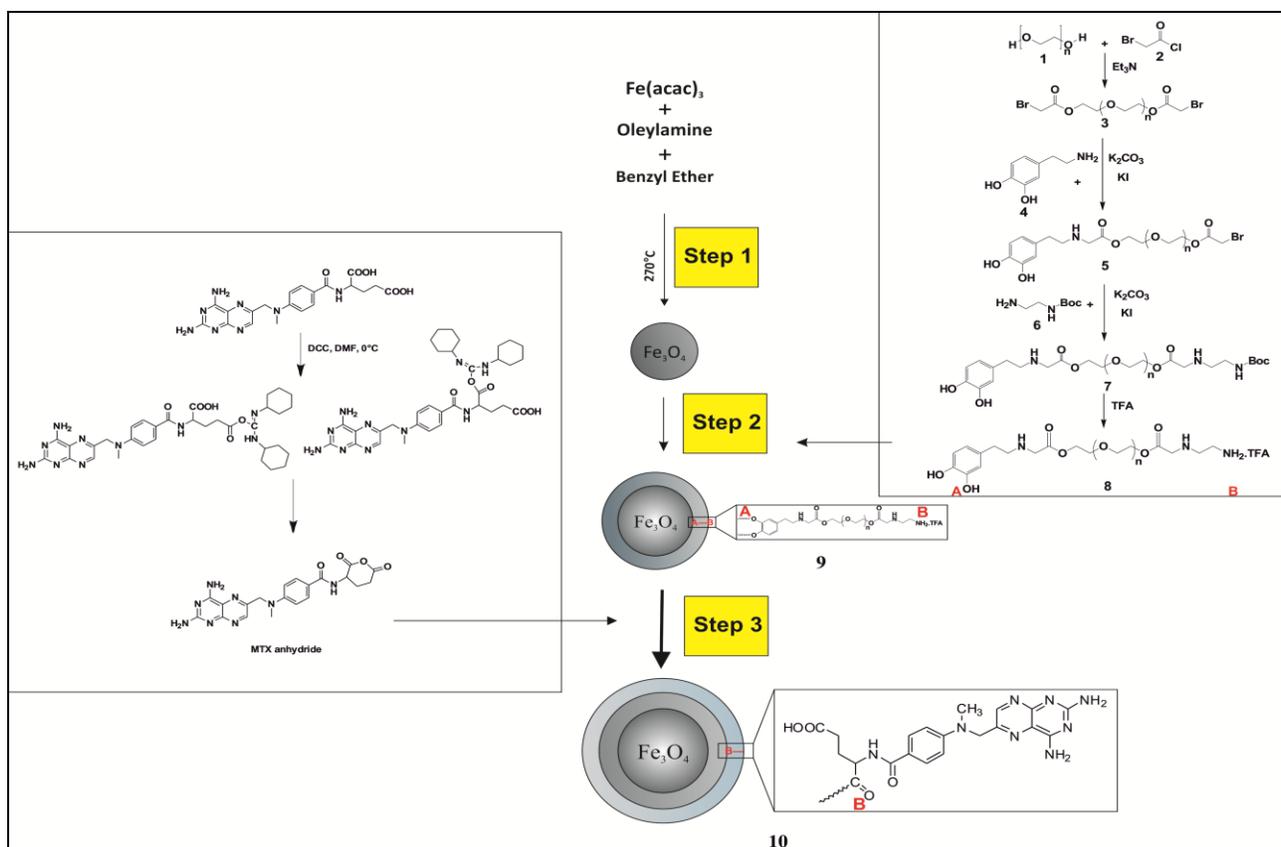


FIG.1: SCHEMATIC STEPS OF SURFACE MODIFICATIONS OF Fe₃O₄. STEP 1: SYNTHESIS OF MNPs. STEP 2: PEGYLATION OF MNPs. STEP 3: CHEMICAL LOADING OF METHOTREXATE.

Analysis of carboxylate group activation of MTX:

To synthesize mono- and di-methylester of MTX, first MTX (3.75 mg, 0.00825 mmol) and DCC (3.7 mg, 0.018 mmol) were dissolved in 1 mL DMF (ratio 1:2.2) and reaction solution was stirred overnight at 0°C. The precipitate was removed by centrifugation at 5000 rpm (10 min, 4°C)¹⁷. After that, the supernatant was divided into two parts. In the first reaction, 0.36μL of dry methanol (1:1.1) was added to one part of the supernatant and the solution was stirred for 3h at room temperature. On the second reaction, the second part of the

supernatant was added to dry methanol (0.7μL) (1:2.2), and the solution was stirred for 3 h at room temperature. For purification of these products, the reaction mixtures were centrifuged separately at 4000 rpm (10 min) and the supernatant was precipitated with 5 mL of diethyl ether. The HPLC profiles (Waters, MA) of two mentioned reaction were monitored by a C18 chromatography column (Waters Spheris orb, 4.6×250 mm) with 30% methanol (V/V) in ammonium phosphate solution (pH 5) and a flow of 1mL/min (15 min, 302 nm).

Characterization of MNPs:

Size of the MNPs was analyzed using a particle

size analyzer, Malvern Nano ZS (Malvern Instruments, Malvern, UK). The size and morphological studies of MNPs were performed with field emission scanning electron microscopy (FESEM) (Mira 3-XMU) and transmission electron microscopy (TEM) (LEO 906, Carl Zeiss, Germany). The surface modifications of MNPs were validated by FT-IR spectroscopy using Shimadzu IR PRESTIGE 21 spectrophotometer (Shimadzu Scientific Instruments, Tokyo, Japan). The XRD signature and average size of MNPs were analyzed at the rate of scan of 0.02° and voltage of 40 kW and 30mA using Scherrer's equation (Siemens D5000 X-Ray Diffractometer, Munich, Germany).

Drug loading and drug release evaluation:

The extent of MTX in PEGylated MNPs was estimated by quantitative ultraviolet (UV) (CECIL, CE1021) spectrophotometric analysis at 302 nm. To evaluate enzymatic and non-enzymatic release of MTX conjugated on MNPs and to simulate intracellular conditions, 0.25 mg/mL of Fe_3O_4 -DPA-PEG-MTX was dispersed in three buffer solution (sodium acetate/acetic acid (pH 3.8, 37°C), phosphate buffered saline (PBS) (pH 7.4, 37°C) and sodium bicarbonate/sodium carbonate (pH 8.8, 37°C)) with 1.25 mg/mL pure protease (Sigma) and without protease enzyme. Incubation was continued for 72 h and released MTX from PEGylated MNPs was quantified by UV spectroscopy at 302 nm.

Cell culture and cytotoxicity analysis: MTT assay:

Both human breast cancer MCF-7 cells (FR-positive cells)^{22, 23} (Pasture Institute, Tehran, Iran) and human lung cancer A549 cells (FR-negative cells) (Pasture Institute, Tehran, Iran) were cultured at a seeding density of 4.0×10^4 cells/cm² into the T-50 flasks using RPMI 1640 media (Gluta MAX) supplemented with 10% FBS (Gibco), 100 units/mL penicillin G, and 100 mg/mL streptomycin (Gibco). The cultured cells were kept at 37°C in a humidified CO_2 incubator during cultivation and during experiments.

Cytotoxicity assay was conducted in both MCF-7 cells and A549 cells. Cells (1×10^4 cells/well) were cultivated onto 96-well plates. At 40–50% confluency (24 h post-seeding), the cultured cells

were treated with different concentrations (i.e. ranging from 0.8 to $16 \mu\text{M}$) of Fe_3O_4 -DPA-PEG-MTX and MTX alone. The concentration of Fe_3O_4 -DPA-PEG-MTX NPs was set to be equivalent to the MTX alone. The treated cells were incubated for different time frames (i.e. 24, 48, and 72 h), and then subjected to (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Merck). The media was removed and 150 mL of fresh media plus 50 mL MTT solutions (prepared as 2 mg/mL in FBS) were added to each well and incubated for 4 h at 37°C in a CO_2 incubator. The media was removed and the cells were washed ($3 \times$), and then the formed formazin crystals were dissolved by adding DMSO ($200 \mu\text{L}$) and Sorenson's buffer ($25 \mu\text{L}$) to each well. The absorbance was read at 570nm using a spectrophotometer (Bio Tek Instruments, Inc., Bad Friedrich shall, Germany).

RESULTS AND DISCUSSION: Engineering of Fe_3O_4 -DPA-PEG-MTX NPs was started via the synthesis of Fe_3O_4 NPs at 270°C by thermal decomposition reaction of $\text{Fe}(\text{acac})_3$ in the presence of oleylamine as a reducing, capping, and monodisperse reagent^{2, 19, 24}. This method produced magnetic carriers with an average size of about 7 nm, which was determined using particle size analyser (**Fig.2**)². To remove water from reaction environment, the reaction mixture was dried using Dean–Stark apparatus at 120°C ²⁵. For improved biostability and better pharmacokinetic properties, MNPs, as nano-scaled carriers, can be further modified with hydrophilic polymers such as dextran, dendrimers, polyvinyl pyrrolidone, PEG, and inorganic layers of gold or silica^{8, 26, 27}. Of these modified MNPs, the PEGylated MNPs are considered as stealth hydrophilic macromolecular nanocarriers^{5, 21}, which can be further decorated through “grafting-onto” approach²⁸. Therefore, DPA-PEG-NH₂ was used for the PEGylation of MNPs.

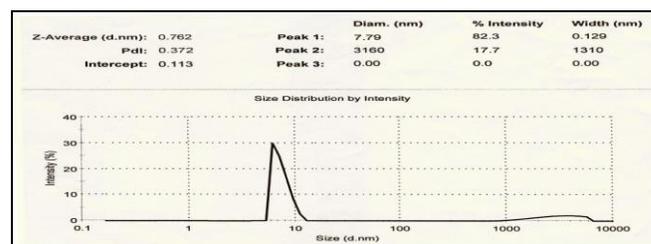


FIG.2: PARTICLE SIZE ANALYSER CURVE OF Fe_3O_4 NPs. AVERAGE SIZE IS ABOUT 7 nm

To synthesize DPA-PEG NH₂, in the first step BBrAC-PEG was synthesized by reacting an excess amount of bromoacetylchloride with PEG in the presence of triethylamine as a base. In the second step, the reaction of BBrAC-PEG with one equivalent of dopamine yielded DPA-PEG-BrAc. In the third step, the DPA-PEG-BrAc was treated with one equivalent of *N*-*tert*-butoxycarbonyl-1, 2-ethylenediamine to give DPA-PEG-NHBoc. Boc (*N*-*tert*-butoxycarbonyl) was removed via trifluoroacetic acid (TFA) to gain DPA-PEG-NH₂². The surface of Fe₃O₄ was made biocompatible using dopamine moiety in DPA-PEG-NH₂ as an anchoring agent. The oleylamine as a capping agent on the surface of MNPs could be replaced with DPA-PEG to gain more hydrophilic and stealth Fe₃O₄ nanocarriers.

As shown in the FTIR spectrum (**Fig.3B**) of Fe₃O₄-DPA-PEG-NH₂, in addition to the peaks related to Fe₃O₄ (**Fig. 3A**) and DPA-PEG, an absorption peak in $\nu_{\max}/\text{cm}^{-1}$ 1487 cm⁻¹ confirms NH₂ available at the end of the structure of the Fe₃O₄-DPA-PEG-

NH₂^{2, 19}. On the other hand, modification of Fe₃O₄ NPs with DPA-PEG-NH₂ can be useful for the conjugation of other functional group onto MNPs. MTX as a targeting and therapeutic agent has been shown to increase passive and active targeting of FRs on tumor cells^{29, 30}. For this purpose, the PEGylated MNPs were conjugated chemically with MTX, a homing device.

To this end, carboxyl groups of MTX were activated via DCC in dry DMF and in the absence of *N*-hydroxysuccinimide for a controlled loading of MTX to Fe₃O₄-DPA-PEG-NH₂¹⁷. MTX was reacted with 1.1 equiv. of DCC for 18 h at 0°C. The white *N,N*-dicyclohexylurea was precipitated by centrifugation and then, activated MTX was chemically reacted with nucleophilic amino group of Fe₃O₄-DPA-PEG-NH₂ to form Fe₃O₄-DPA-PEG-MTX¹⁷. The MTX loaded MNPs were easily collected by magnetic bead separation system. The UV spectrophotometry of unreacted MTX in 302 nm revealed about 88% loading efficiency of drug onto magnetic nanocarriers.

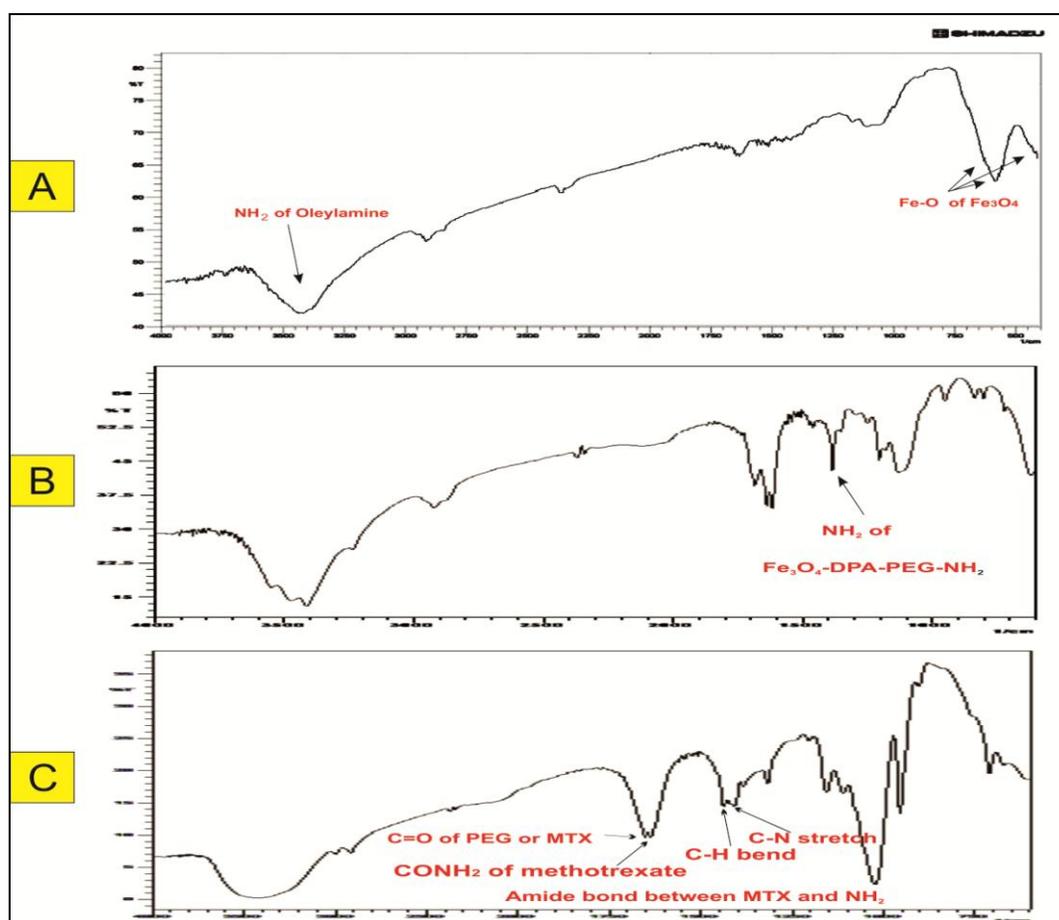


FIG.3: FT-IR SPECTRA OF (A) Fe₃O₄ MNPs , (B) Fe₃O₄ -DPA-PEG-NH₂ AND (C) Fe₃O₄-DPA-PEG-MTX; ABSORPTION PEAK IN ν_{\max} 1648 cm⁻¹ IS RELATED TO NEW AMIDE BOND OF METHOTREXATE.

To explain the activation method and find more active carboxyl group of MTX in a selective condition, MTX was incubated with 2.2 equiv. of DCC in DMF and stirred overnight at 0°C. The activated MTX was reacted with two different equivalent of methanol for 3h at room temperature. The HPLC analysis of these different methylesters

showed that in the presence of 1.1 equiv. of methanol, α -methylester of MTX was the major product (**Fig. 4A**). On the other hand, by 2.2 equiv. of methanol, dimethylester was the selective product (**Fig. 4B**).

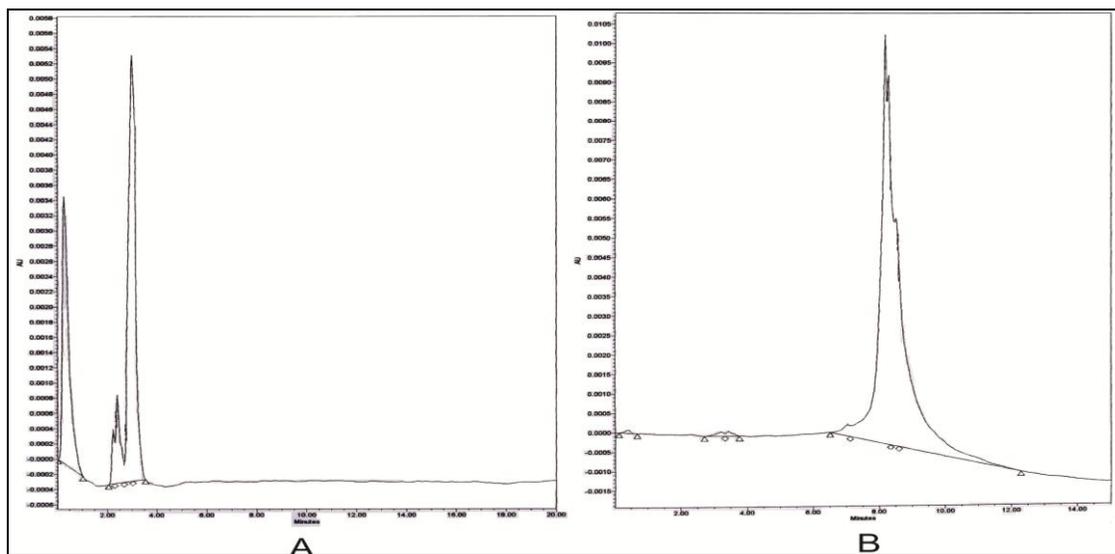


FIG.4: HPLC ANALYSIS OF THE PRODUCT OF THE REACTION OF ACTIVATED METHOTREXATE WITH METHANOL. (A) A-METHYLESTER OF MTX IS MAJOR PRODUCT. (B) DIMETHYLESTER IS SELECTIVE PRODUCT

In the FTIR spectrum of Fe_3O_4 -DPA-PEG-MTX, the ν_{max} 1430 cm^{-1} (C–H bend) and 1390 cm^{-1} (C–N stretch) increased, which confirmed the presence of amide bond between MTX and NH_2 at the end of PEGylated MNPs. Also ν_{max} 1648 cm^{-1} is related to new amide bond of MTX (**Fig.3C**). **Fig. 5** represents the FESEM and TEM micrographs of

Fe_3O_4 -DPA-PEG-MTX showing an average size of 37nm. The X-ray diffractometry of Fe_3O_4 -DPA-PEG-MTX showed the cubic structure with an average size of 37 nm. It also showed that surface modification of Fe_3O_4 NPs did not lead to any crystal phase changes. **Fig. 6** represents the XRD pattern of Fe_3O_4 -DPA-PEG-MTX.

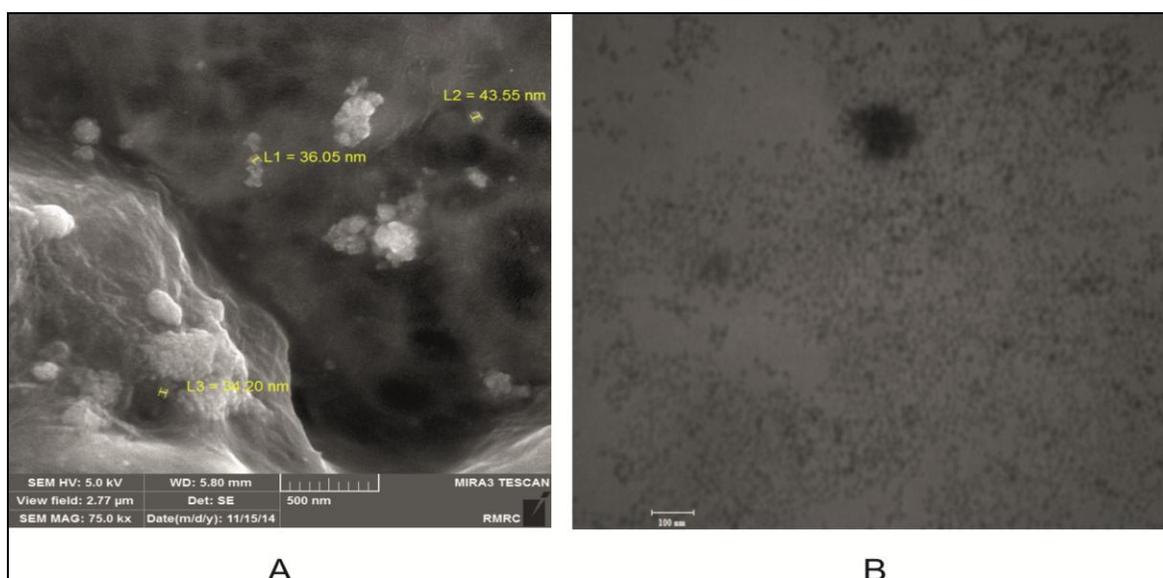


FIG.5: (A) FIELD EMISSION SCANNING ELECTRON MICROSCOPY (FESEM) OF Fe_3O_4 -DPA-PEG-MTX; (B) TRANSMISSION ELECTRON MICROSCOPY (TEM) MICROGRAPHS OF Fe_3O_4 -DPA-PEG-MTX

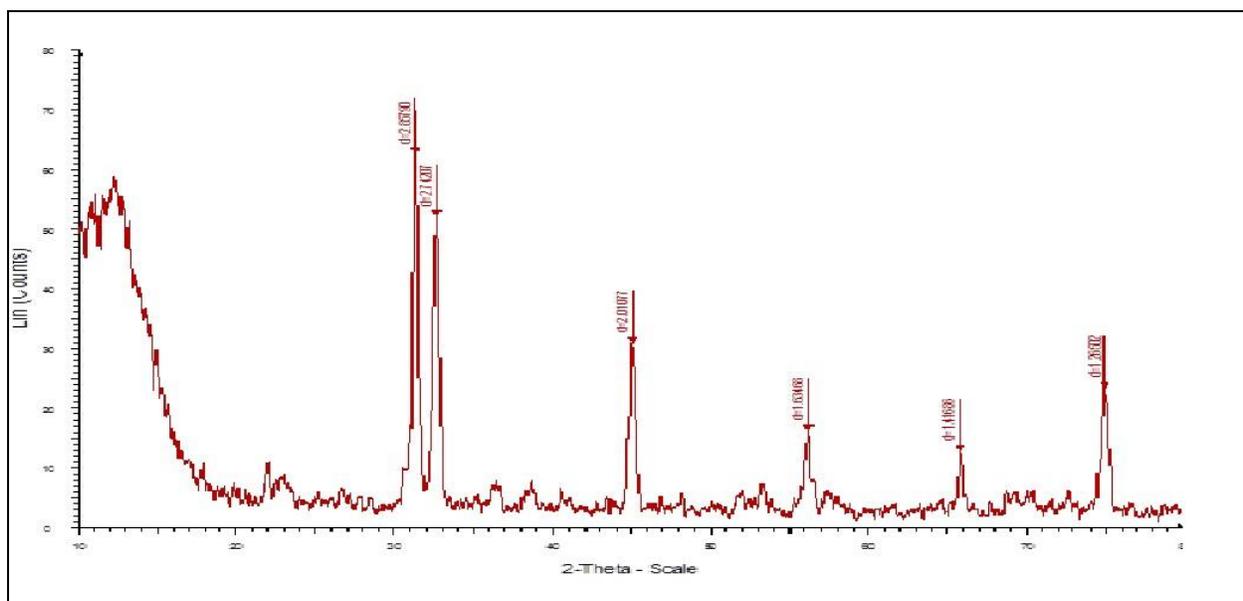


FIG.6: THE X-RAY DIFFRACTOMETRY OF Fe_3O_4 -DPA-PEG-MTX NPS.

The uptake model of MTX-conjugated MNPs into target cells is vesicular machineries via receptor-mediated endocytosis. It was assumed that the proteases in the lysosomal vesicles could cleave the amide bond between MTX and MNPs^{1, 31}. So, it suggested that the mechanism of MTX releasing from MNPs could be an enzyme-dependent manner that in this process, proteases at lower pH hydrolyzed MTX from modified MNPs. Also, we expect that this covalent bond could be secure in blood circulation condition. To simulate this mechanism, the drug loaded MNPs were placed in the dialysis bag and incubated in both protease and

non-protease solution at three pH conditions (3.8, 7.4, and 8.8) in 37°C. The details of pH and enzyme-dependent releases in 72 h are given in Fig.7. The released MTX was quantified by UV absorption in 302 nm. Our preliminary analyses have confirmed the enzyme dependent release of MTX; the highest rate of drug release from the nanoparticles was observed at pH 3.8 with protease enzyme (85.12%) in which the enzyme was in the most active form. In addition, the data showed that after 72h, in the absence of enzyme, the release percentage of drug was very low (in all three buffers).

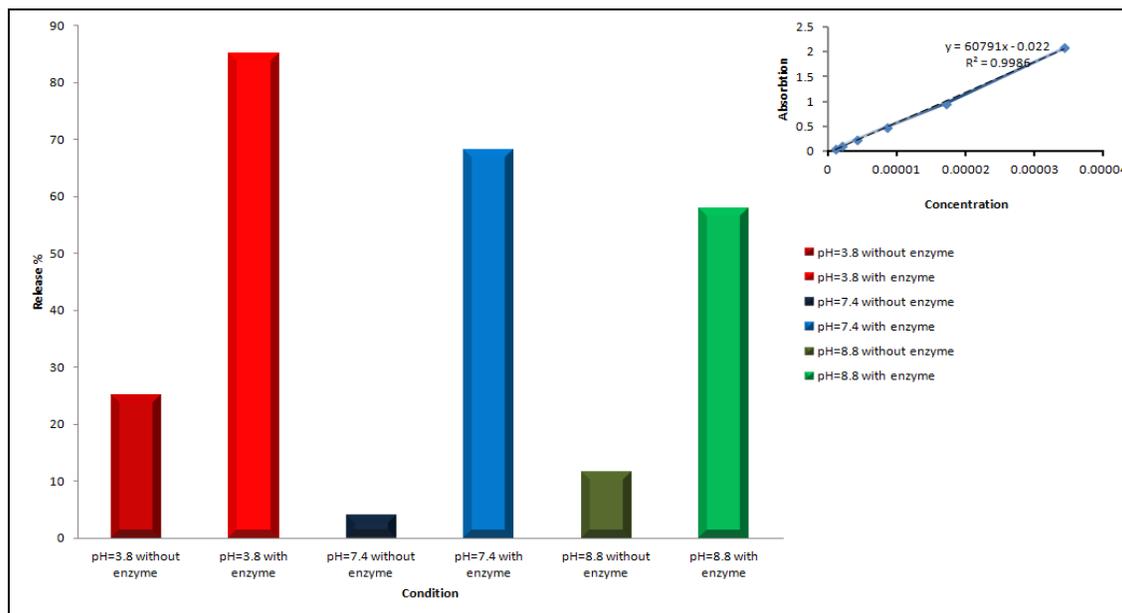


FIG.7: THE RELEASE STUDY OF MTX LOADED MAGNETIC NANOPARTICLES; THE HIGHEST RATE OF DRUG RELEASE FROM THE NANOPARTICLES WAS OBSERVED AT pH 3.8 WITH PROTEASE ENZYME.

We selected MCF-7 (FR-positive) and A549 (FR-negative) cell lines to evaluate the uptake of MTX conjugated MNPs by FR. MTT assay results showed that MTX-loaded MNPs could have a time and dose response to FR-positive MCF-7 cell lines in which Fe₃O₄-DPA-PEG-MTX NPs could be actively taken up by MCF-7 cells while the cytotoxic effect showed an enzymatic cleavage of MTX from MNPs. In the FR-positive MCF-7, we observed a significant difference of cytotoxicity between 24, 48, and 72 h (about 80% toxicity after

72h, **Fig. 8C**). On the other hand, FR-negative A549 cells could not actively take up the MTX-conjugated MNPs and we did not observe a significant cytotoxicity effect (**Fig. 8D**). In MCF-7 cells, IC₅₀ values of free MTX and Fe₃O₄-DPA-PEG-MTX were appraised 4 μM and 1.7 μM, respectively. As shown in **Fig. 8A–C**, Fe₃O₄-DPA-PEG-MTX demonstrated a trend of time and concentration-dependent inhibitory effects.

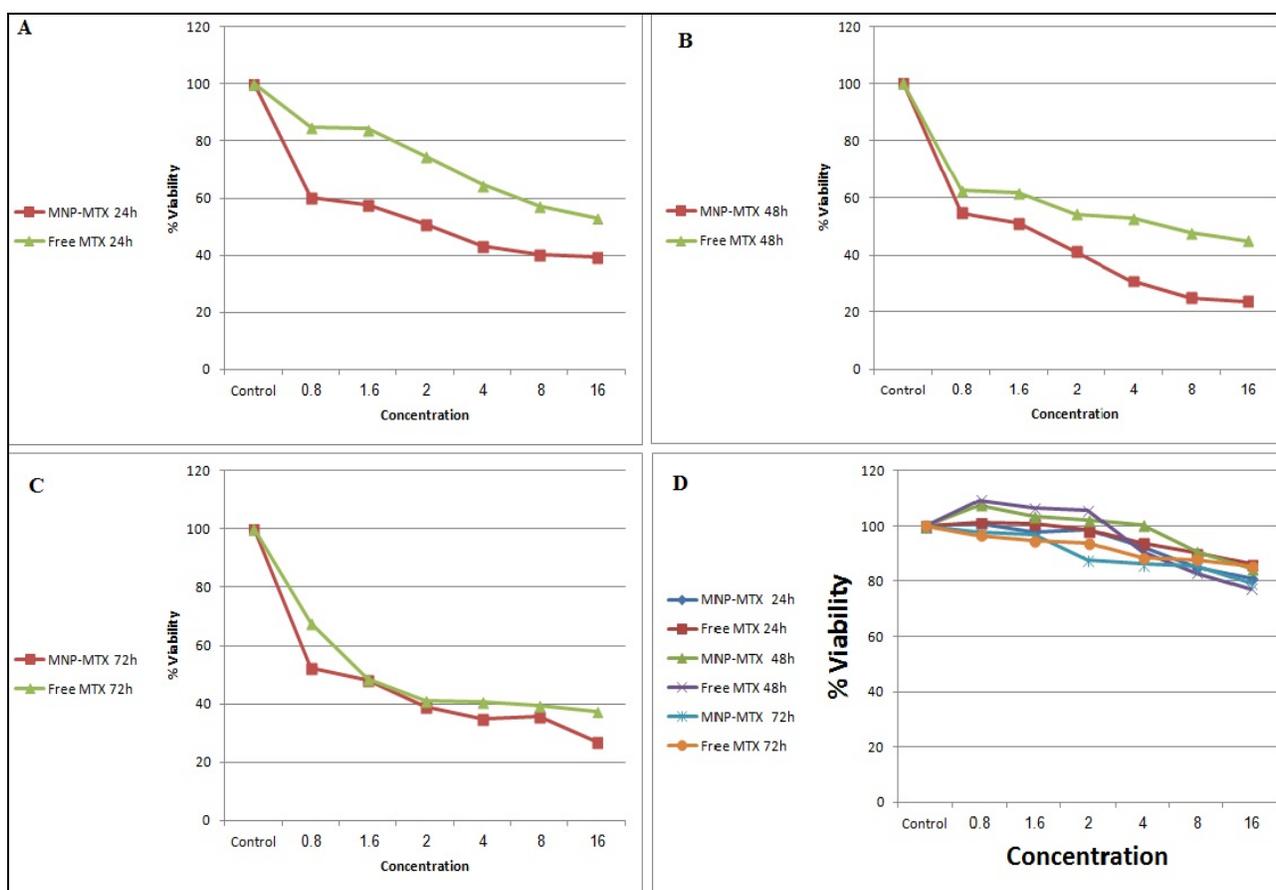


FIG.8: CYTOTOXIC STUDY (MTT ASSAY) OF Fe₃O₄-DPA-PEG-MTX NPs AND PURE METHOTREXATE IN THE FR-POSITIVE MCF-7 AND FR-NEGATIVE A549 CELLS. (A) TREATED MCF-7 CELLS AFTER 24H. (B) TREATED MCF-7 CELLS AFTER 48H. (C) TREATED MCF-7 CELLS AFTER 72H. (D) TREATED A549 CELLS AFTER 24, 48 AND 72H.

CONCLUSIONS: Hitherto, we showed successful synthesis of the PEGylated MNPs conjugated with MTX, which exhibited significant inhibitory effects in the FR expressing MCF-7 cancer cells. Also, our analyses have confirmed enzyme-dependent release of MTX in acidic condition (conditions close to the physiological environment of the cell). Thus, we could suggest these targeted MNPs as bifunctional nanocarriers for synchronous targeting and therapy of different types of FR positive malignancies.

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