Combined chemo-magnetic field-photothermal breast cancer therapy based on porous magnetite nanospheres

Majid Sharifi1,2, Anwarul Hasan3,4*, Nadir Mustafa Qadir Nanakali5,6, Abbas Salihi7, Fikry Ali Qadir7, Hawzheen A. Muhammad8, Mudhir Sabir Shekha7, Falah Mohammad Aziz7, Karwan M. Amen9,10, Farrokh Najafi11, Hasan Yousefi-Manesh12 & Mojtaba Falahati1*

The efficacy of different modalities of treating breast cancer is inhibited by several limitations such as off-targeted drug distribution, rapid drug clearance, and drug resistance. To overcome these limitations, we developed Lf-Doxo-PMNSs for combined chemo-MF-PTT. The PMNSs were synthesized by hydrothermal method and their physicochemical properties were examined by FE-SEM, TEM, DLS, TGA, XRD investigations. The cytotoxicity of as-synthesized NPs against 4T1 cells was carried out by MTT and flow cytometry assays. Afterwards, the anti-cancer activities of as-synthesized Lf-Doxo-PMNSs on the tumor status, drug distribution and apoptosis mechanism were evaluated. The anti-cancer assays showed that Lf-Doxo-PMNSs significantly suppressed the cancer cell proliferation and tumor weight by prolonging drug availability and potential drug loading in tumor cells; whereas they showed a minimum cytotoxicity against non-cancerous cells. Likewise, combined chemo-MF-PTT using LF-Doxo-PMNSs displayed the highest anti-cancer activity followed by combined chemo-PTT and combined chemo-MF therapy based on altering the apoptosis mechanism. Therefore, these results showed that combined chemo-MF-PTT based on Lf-Doxo-PMNSs can be used as a promising therapeutic platform with potential targeted drug delivery and high loading capacity features as well as reducing cancer drug resistance.

Breast cancer is one of the most common types of malignancies and is the first leading cause of death in women1. The use of combined therapeutic platforms has provided promising outcomes for the treatment of breast cancer. In this regard, it has been attempted to utilize nanomaterials to develop theranostic platforms for the treatment of breast cancer2. However, working in various therapeutic areas based on nano-based platforms is a complex and difficult process due to the limited colloidal stability and combined-modality therapy, toxicity, and complex mechanism of NPs in the biological systems3–5. A better understanding of breast cancer microenvironment, cancer drug resistance, control of drug clearance and drug delivery along with auxiliary therapeutic platforms such as chemotherapy, MF therpay, PTT or their combination have been facilitated the treatment of breast cancer6–8. Therefore, the use of targeted NPs particularly magnetic NPs by combination of chemotherapy and thermal or optical therapy in the breast cancer model can induce fewer side effects than the conventional methods such as radiotherapy and chemotherapy. In this line, IONS is known as one of the most important drug delivery vehicles

1Department of Nanotechnology, Faculty of Advanced Sciences and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran. 2Department of Animal Science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran. 3Biomedical Research Center, Qatar University, Doha, 2713, Qatar. 4Department of Mechanical and Industrial Engineering, College of Engineering, Qatar University, Doha, 2713, Qatar. 5Department of Biology, College of Science, Cihan University-Erbil, Kurdistan Region, Iraq. 6Department of Biology, College of Education, Salahaddin University-Erbil, Kurdistan Region, Iraq. 7Department of Biology, College of Science, Salahaddin University-Erbil, Kurdistan Region, Iraq. 8Department of Microbiology, College of Medicine, University of Sulaimani, Sulaimani, Kurdistan region, Iraq. 9Department of Medical Analysis, Faculty of Science, Tishk International University, Erbil, Iraq. 10College of Nursing, Hawler Medical University, Erbil, Iraq. 11Department of Biomaterial engineering, University of Amir-Kabir, Tehran, Iran. 12Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran. *email: ahasan@qu.edu.qa; mojtaba.falahati@alumni.ut.ac.ir
due to some novel and promising therapeutic characteristics such as inert nature, cost-effective, and biocompatibility\(^{6,8}\). Also, they show the ability to be used as an outstanding agent in combined modality approach for cancer treatment\(^{11}\). For instance, in a mice model, Yang, et al.\(^{19}\) found that hyaluronic acid-modified IONSs containing CD44 antibodies were able to decrease the activity and volume of breast cancer tumors up to 2-fold via PTT (808 nm laser at 1 W/cm\(^2\)) in addition to 40% enhancement of magnetic resonance imaging (MRI) of breast cancer tumors. Likewise, Wang, et al.\(^{11}\) using transferrin-coated IONSs not only reduced the toxicity of the synthesized NPs, but also increased the imaging contrast similar to the long circulation of NPs in the blood. However, the low surface of IONSs for drug loading as well as the need of combined-modality therapy encouraged the researchers to utilize PMNSs as a potential agent in breast cancer therapy.

The use of PMNSs in photothermal therapeutic platforms has been intensively developed, because these NPs are capable of absorbing magnetic and optical wavelengths that are not commonly absorbed in biological systems. Indeed, the heat induced by absorbing light results in some cellular responses like apoptosis through elevating ROS level\(^{6,12}\). PMNSs can locally destroy the target site by hyperthermia during MF or PTT activities without inducing significant side effects against adjacent tissues\(^2\). However, very few studies have been done for application of PMNSs in the development of anti-cancer platforms compared to other forms of iron NPs. While, the use of PMNSs can result in increased drug loading as well as reduction of the dosage of the drugs, improved magnetic intensity, and single injection of NPs\(^3\). For instance, in a cellular model, Su, et al.\(^5\) by producing Lf-modified PMNSs not only provided higher efficacy in the treatment of breast cancer with a lower dose of paclitaxel along with MF therapy, but also enhanced imaging quality of breast cancer cells.

On the other hand, some limitations in targeted drug delivery\(^{15}\) result in severe toxicity to liver, kidney and spleen\(^{13–17}\), as well as the poor distribution of anti-cancer drugs in tumor site\(^{6,10}\). Therefore, several strategies like development of some NP-based platforms for targeted drug delivery have been reported\(^{11,17,18}\). In this regard, it has been well documented that protein coating of NPs can enhance active absorption of a loaded drug by the tumor cells via protein receptors\(^9,20\). Since Lf is found in exocrine secretions such as saliva, milk, and gastrointestinal secretions\(^{21}\), its application may increase the presence of this compound in the ducts. Also, the use of Lf as a coating agent due to its accumulation in breast tissue can be effective in targeting breast cancer\(^{22}\). In several studies, the anti-cancer activity of Lf is associated with the capacity of apoptosis through down-regulation of Bcl-2 mRNA\(^{23}\), along with up-regulation of the pro-apoptotic Bax and Caspase 3 mRNA\(^{24}\). Besides, Lf can result in targeted delivery of NP containing Doxo to tumor site\(^{25}\) as well as an increase in the blood circulation time due to ability to escape from mononuclear phagocyte system (MPS)\(^{26}\) and controlling drug release\(^1\), whereas, the anti-cancer mechanism of Lf is not fully understood.

Herein, in addition to the synthesis of Lf-Doxo-PMNSs by hydrothermal methods, we examined the physicochemical properties of the NPs followed by the toxicity assay of the NPs and drug bio-distribution in vitro and in vivo. Also, in addition to the blood clearance of as-synthesized platform and its level in tumor and different tissues, we investigated the drug release profile in the different pH. Furthermore, the effects of NPs on the morphological changes of tumor cells and off-targeted tissues were investigated by histological assay. Finally, the molecular mechanism of combined-modality therapy using Lf-Doxo-PMNSs on tumor cell death was evaluated by molecular assays.

Material and methods

Materials. Ferric chloride, sodium acrylate, sodium acetate, ethylene glycol (EG), diethylene glycol (DEG), tetraethyl orthosilicate (TEOS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxy succinimide (NHS), dimethyl sulfoxide (DMSO), and poly-acrylic acid (PAA) were obtained from the Merck Company (Germany). Dulbecco’s Modified Eagle Medium (DMEM) cell culture, MTT powder, and fetal bovine serum (FBS) were purchased from Gibco (Scotland). Lf and Doxo were purchased from the Sigma Aldrich Company (USA). Annexin V-FITC kit was obtained from Iq products, Poland. The BALB/c mice and 4T1 cells were purchased from Pasteur Institute (Tehran, Iran).

Synthesis of PMNSs and Lf-PMNSs. Based on the modified method described by Xuan, et al.\(^{27}\), ferric chloride (0.54 g), sodium acrylate (1.5 g), and sodium acetate (1.5 g) were dissolved in EG and DEG (total volume: 20 mL with equal proportion) under magnetic stirring. The gained homogeneous yellow solution moved to a Teflon-lined stainless-steel autoclave and heated at 220 °C. After 10 h, the autoclave cooled to 25 °C and the obtained PAA-coated MNSs were washed several times with ethanol and water and dried in vacuum for 15 h.

Then, PAA-coated MNSs were covered by SiO\(_2\) layer. Briefly, 5 mL of aqueous solution of PAA-coated MNSs diluted with 1 mL of water and 60 mL of ethanol. The mixture was homogenized by ultrasonication for 40 min before adding 2 mL of ammonia solution. After 40 min, 0.2 mL of TEOS solution diluted in 10 mL of ethanol was injected and the reaction was carried out for 120 min and the final product was gathered with the magnet.

Then, nanospheres were washed several times with ethanol and water, followed by drying in vacuum for 15 h. Afterwards, the MNSs coated with SiO\(_2\) were heated in an oven with a heating rate of 5 °C/min to reach 700 °C for 5 h. After cooling at room temperature, a product of the Fe\(_3\)O\(_4@\)SiO\(_2\) NSs was produced, followed by dissolving in a 0.5 M NaOH for 7 h under mechanical shaking. Finally, the NPs were gathered by magnetic methods and washed several times with water and ethanol.

To provide carboxyl linkers for attachment of Lf to the PMNSs as a bio-gate, 0.5 mg of PMNSs was sonicated in 250 μL solution prepared by 10 mg/mL of EDC, 250 μL of NHS (10 mg/mL), and 7 μL of NaOH (1 M) for 15 min. Extra EDC and NHS were eliminated by using PD-10 column equilibrated with 10 mM phosphate buffered-saline solution (PBS, pH 7.4). Then, 250 μL Lf (1 mg/mL) was added to the activated PMNSs and maintained overnight at 25 °C. Finally, centrifugation (5000 g, at 5 min) was run to separate the synthesized Lf-PMNSs.2
Drug loading and releasing. The fluorescence spectra (Hitachi F 2500 spectrophotometer) were used to determine the loading capacity of Doxo. For this purpose, 500 μg of PMNSs and Lf-PMNSs were added to Doxo solution at different concentrations of 100, 200, 300, 400 and 500 μg, and mixed thoroughly by shaking at room temperature for 24 h. The nanocarriers containing the Doxo were then separated from the solution using a magnet. The loading efficiency (%) was then determined by the following Eq. 1:

\[
\text{Loading efficiency(\%)} = \frac{A - B}{B} \times 100
\]

Where A is the total amount of Doxo, and B is the amount of Doxo remaining in the solution.

After evaluating the loading efficiency (%), 2.5 mg of PMNSs or Lf-PMNSs was added to 2.5 mL of DMSO solution containing 2.5 mg of Doxo to load the drug into the carriers. Then, the Doxo-nanocarriers were dried under vacuum to evaporate the DMSO for 24 h. Finally, PBS was used to wash the carrier three times to remove unloaded Doxo.

The in vitro drug release was probed at 37 °C for 150 min by the incubation of the Doxo-PMNSs and Lf-Doxo-PMNSs in PBS at different pH (2.5, 5.0 and 7.5). Briefly, certain amounts of NPs were dissolved in 10 mL of PBS and purification was done by dialysis process (MWCO 3500) against 40 mL of the same buffer at 100 rpm. At prescribed time intervals, 5 mL of solution was extracted for the measurement by absorbance at 480 nm and was restored by an equal volume of the same buffer solution. The Doxo cumulative release was estimated based on the following Eq. 2:

\[
\text{Cumulative drug release (\%)} = \frac{5 \times \sum_{i=1}^{n} C_{i} + 50 \times C_{n}}{\text{weight of Doxo on PMNSs}} \times 100
\]

Where, \(C_{i}\) and \(C_{n}\) refer to the of Doxo concentration at time i and n, respectively.

Characterization of NPs. The morphologies of Lf-Doxo-PMNSs were depicted by applying a FESEM (JEOL-6700, Japan). Also, TEM images were captured on a high-resolution transmission electron microscope (HRTEM, JEM-2100) at an accelerating voltage of 100 kV. A high-frequency MF heating of Lf-Doxo-PMNSs was used at 4 and 8 kA/m strengths. After heating, the temperature was evaluated by applying a thermometer. The increase in the temperature of the Lf-Doxo-PMNSs by PTT was recorded by a thermometer during the irradiation with a wavelength of 808 nm.

To measure the surface charges and hydrodynamic size of NP, DLS study was done using a Zetasizer nano series (Worcestershire, United Kingdom). Also, the thermal durability of the fabricated samples was assessed using TGA (Perkin-Elmer, TGA-7) under N2 at a heating rate of 5 °C/min in the range of 50–650 °C. The XRD series (Worcestershire, United Kingdom). Also, the thermal durability of the fabricated samples was assessed using TGA (Perkin-Elmer, TGA-7) under N2 at a heating rate of 5 °C/min in the range of 50–650 °C. The XRD arrays were documented on a Power X-ray Diffractometer (XRD-6000; Japan) with a scan range from 20° to 80° quantum interference device (SQUID, MPMS-XL) was applied from 10,000 to +10,000 G at 298 K. Besides, the BET surface area of the sample was investigated using nitrogen adsorption-desorption isotherm modules at 77 K on a nitrogen adsorption apparatus (Micromeritics ASAP). Pore size distribution was considered from desorption branch of the isotherm through the Barrett-Joyner-Halenda process by applying the Halsey equation.

In vitro model. The 4T1 cells were cultured in DMEM medium with 10% PBS, 100 U/mL penicillin, and 100 μg/mL streptomycin and maintained in an incubator with 5% CO₂ at 37 °C and 95% humidity. The cells were trypsinized with 0.25% trypsin-EDTA, and re-suspended in DMEM medium for further experiments.

Cell viability. The 4T1 cells were seeded at 30,000 cells/well into 96-well plate and treated with different concentrations of PMNSs, LF-PMNSs, Doxo-PMNSs and Lf-Doxo-PMNSs (5–20 μg/mL), and free Doxo (2.5–10 μg/mL), and incubated for 24 h. The medium was then removed by PBS and 0.5 mg/mL of MTT solution was added for 4 h (50 μL per well). After eliminating medium, the residual crystals were dissolved in 100 μL of DMSO and the plates were shaken gently for 2 min. Then, cell viability was estimated at λ = 570 nm. The % cell viability was estimated by using the Eq. 3:

\[
\% \text{ Cell viability} = \left( \frac{\text{NS}_{\text{abso}}}{\text{NC}_{\text{abso}}} \right) \times 100
\]

Where, \(\text{NS}_{\text{abso}}\) is the individual absorbance of the free Doxo and PMNSs, Lf-PMNSs, Doxo-PMNSs and Lf-Doxo-PMNSs-loaded well and \(\text{NC}_{\text{abso}}\) is the mean absorbance of control well.

Apoptosis and ROS assays. Flow-cytometry assay was used to analyse the percentage of apoptotic and necrotic cells. Based on the method described above, 4T1 cells (3 × 10⁵ cells per well) were plated into 6-well plate and cultured with free Doxo (7.5 μg/mL), PMNSs (15 μg/mL), Lf-PMNSs (15 μg/mL), Dextoxo-PMNSs (15 μg/mL of NPs with 7.5 μg/mL of Doxo), and Lf-Doxo-PMNSs (15 μg/mL of NPs with 7.5 μg/mL of Doxo) in incubator for 24 h. Then, the cells were collected with 3000 g centrifugation for 3 min, washed with cold PBS, and were resuspended in 100 μL of Annexin V-FITC binding buffer (HEPES buffer: 0.1 M, NaCl 1.4 M, CaCl₂ 25 mM, pH 7.4). Then, 2 μL of Annexin V-FITC conjugated Alexa Fluor 488 was added to the cell solution for 15 min at room temperature. Also, 400 μL of binding buffer and 5 μL of propidium iodide were added to the solution and the samples were analysed by using FACscan (BD Bioscience, USA).

To evaluate intracellular ROS, 3 × 10⁵ cells per well was cultured into 6-well plate in the presence of free Doxo (7.5 μg/mL), and PMNSs, Lf-PMNSs, Doxo-PMNSs, and Lf-Doxo-PMNSs (15 μg/mL) for 24 h. Also, 10 μM 2′, 7′-dichlorofluorescein was added per well of treated cells and kept at 37 °C with 5% CO₂ for 1 h, followed by twice
warming with PBS. Then, 500μL of PBS was added to each well and homogenized carefully. Ultimately, the fluorescent intensity was evaluated by applying FACSscan (BD Bioscience, USA).

**In vivo studies.** For in vivo investigations, 72 mice were divided into 9 groups. 6-weekly BLAB/c mice weighing 22.5 ± 2.3 g were used under standard conditions (at 25°C and 55% of humidity with free access to water and feed) with 12 h of light and darkness. The 4T1 cells (5 × 10^5 cells) were injected subcutaneously at the end of the mammary gland on the left side of the mice. After 18 days, when the tumors were reached to 136.1 ± 9.2 mm^3, the mice were treated with different therapeutic (PMNSs, Lf-PMNSs, Doxo-PMNSs, Lf-Doxo-PMNSs, and free Doxo).

**Statements.** All procedures related to the use of animals were carried out based on the animal care policy adopted by the Declaration of Helsinki (DOH), and its later amendments or Comparable ethical standards. The experimental procedures, the animal use and care protocols were approved by review board committee of Avicenna Research Institute and Tehran University.

**Tumor weight.** The mice every 3 days were frequently checked for abnormal consequence. To measure the relative tumor volume (RTV), the tumor volume (TV) was determined in each measurement by the digital vernier caliper based on Eq. 4, and then RTV was obtained by Eq. 5.

\[ TV(\text{mm}^3) = \frac{1}{2} \times (\text{length} \times \text{width}^2) \]  

\[ RTV = \frac{TV_n}{TV_0} \]  

Where TV_n is the TV on day n and TV_0 is the TV on day 0. Furthermore, mice were sacrificed on day 18, and the tumors collected and weighed.

**Doxo distribution.** HPLC method was used to evaluate the amount of Doxo in the blood and main tissues. In order to determine the level of the drug in the blood, after Doxo injection on day 9, 0.3 mL of blood samples were collected at different time intervals (0.5, 1, 1.5, 3, 6, 12, 24, 48, and 72 h) and then centrifuged at 1600 g at 4°C for 15 min. The plasma was kept at –20°C and the level of Doxo was determined using HPLC (SPD-M20A, Japan). For evaluating the level of Doxo in different tissues, the samples were washed with cold saline and dried on filter paper. Tissues were then homogenized in a blend of acetonitrile and water (50:50) for liquid-liquid extraction described by Zhang, et al. Furthermore, to determine the bio-distribution of Doxo, fluorescence imaging with excitation of 485 nm and emission of 590 nm was performed in the whole body by the FlouVision (CCD camera) after 8 h of drug injection. Likewise, at the end of the experiment, the tissues were analyzed quantitatively by ex vivo fluorescence imaging. All mice and tissues were imaged with the same device settings.

**Magnetic field and photothermal therapy.** To create a high-frequency MF to carry out the heating of the tumor tissue in combined-modality therapy, all mice every three days were anesthetized and placed in the center of the loops for 4 min after 8 h of injection. An AC MF of 4 kA/m amplitude (2 kW, 540 kHz) was used for 4 min. The tumors and tissues were collected and weighed for analyzing on day 18. Also, for PTT assay, all tumors were irradiated with 808 nm laser every 3 days and 8 h after Lf-Doxo-PMNSs injection, through the skin surface with power density of 5 W/cm^2 and spot diameter of 10 mm for 5 min.

**Histological assay.** Formaldehyde (10%) was applied to fix the tissues followed by passage and embedding in paraffin. In order to perform the hematoxylin and eosin (H&E) staining, paraffin blocks were sectioned by 4 μm thickness. Slides were investigated at the microscopic level (Olympus-BX51 microscope) and an Olympus-DP12 camera was applied to obtain digital photos and graded by the Scharff-Bloom-Richardson Scale.

**Gene expression analysis.** Tumors obtained from different groups of Lf-Doxo-PMNSs, combined chemo-MF therapy, combined chemo-PTT, and combined chemo-MF-PTT were digested with collagenase IV (Sigma-Aldrich) for 3 h, and then the single-cell suspension was purified by a density gradient centrifugation. Then, trizol reagent was applied to obtain total RNA from tumors (~80 mg) based on the manufacturer’s protocol. The RNA was then measured at 260–280 nm by applying a UV–VIS spectrophotometer (Eppendorf). To eliminate any contaminant genomic DNA, the RNA was treated with DNase I and cDNA was produced from 1 μg of RNA using revert aid First Strand cDNA Synthesis Kit (Fermentas). Table 1 exhibits the specific primers used for the PCR reaction. Quantitative PCR was executed on an ABI 7500 real-time PCR system (ABI, USA) by applying a SYBR Premix Ex Taq Reagent Kit (Takara) according to the manufacturers’ recipes. The mRNA expression of TNF-α, Bax, and Caspase-3 was measured by 2^−ΔΔCT. The β-actin amplification was used as a housekeeping gene.

**Statistical analysis.** The data is reported as mean values with standard deviation (SD). Statistical analysis was carried out with one-way ANOVA and statistical differences were studied at different levels.

**Results**

**NP characterization.** The size and morphology of the synthesized PMNSs were studied by the FE-SEM (Fig. 1A) and TEM (Fig. 1B) techniques, respectively. Imaging techniques showed that the size of PMNSs and Lf-PMNSs were 106 and 119 nm, respectively, which consists of surface cracks, indicating the presence of the inner cavities. The results of DLS also showed that the thickness of Lf on the PMNSs was approximately 15–19 nm.
Then, photothermal and MF heat generated in PMNSs was described in Fig. 2A,B, respectively. Our results indicated that laser irradiation at 808 nm and MF (4 and 8 kA/m) sufficiently triggered the thermal activation of the PMNSs.

| Gene name | PCR primer sequence 5’ → 3’ | Cycles (no.) | PCR step | Temperature/time |
|-----------|-----------------------------|--------------|----------|-----------------|
| TNF-α     | 5’-GCTTCAACGGCACCCTGACAAT-3’ | 30           | Annealing | 58 °C/30 sec   |
|           | 3’-CTGAGTCTCGGGGTATTGTGCAATT-3’ |              |          |                 |
| Bax       | 5’-CAGGATGCCCTCCAAGGAA-3’    | 30           | Annealing | 58 °C/30 sec   |
|           | 3’-CGTGTCACCGTACAACTCA-3’    |              |          |                 |
| Caspase-3 | 5’-CTTGTGATCTTTGTGGTGG-3’    | 30           | Annealing | 58 °C/30 sec   |
|           | 3’-GATGCTTTCCAAGCTCCTGTG-3’  |              |          |                 |
| β-Actin   | 5’-AATCCATCATGAAGTGTA-3’     | 25           | Annealing | 56 °C/30 sec   |
|           | 3’-ACTCTGCTTGGATGATCCAC-3’   |              |          |                 |

Table 1. Primer sequences for real time-PCR.

Figure 1. (A) FE-SEM and (B) TEM images of Lf-Doxo-PMNSs.

Then, photothermal and MF heat generated in PMNSs was described in Fig. 2A,B, respectively. Our results indicated that laser irradiation at 808 nm and MF (4 and 8 kA/m) sufficiently triggered the thermal activation of the PMNSs.
The particle size histogram (Fig. 2C) was clarified by the DLS analysis showed that the size distribution of PMNSs and Lf-PMNSs were between 60 to 175 nm, and 70 to 190 nm with a polydispersity index (PDI) of 0.151 and 0.446, respectively. So, this data determined that by attachment of Lf, the size of Lf-PMNS increases between 10 to 19 nm (from ~118 ± 2.65 to ~130 ± 1.48 nm). In addition to imaging and DLS, TGA technique was used to confirm the loading of Lf and Doxo on the NP surface. Figure 2D shows that the samples have a slight weight loss.
due to the loss of water at temperatures between 70 to 100 °C. By rising the temperature up to 360 °C, the weight loss (10–12% approximately) of the samples appears to be due to the breakdown of Lf and Doxo. These data display that Lf and Doxo have been successfully loaded on the PMNS surface.

XRD patterns exhibited that the crystalline phase of PMNSs is magnetic (Fig. 2E). Six main diffraction peaks at 2θ = 30° (220), 36° (311), 44° (400), 53° (422), 57° (511), and 63° (440) can be discovered according to magnetic crystal structure of fabricated nanoconjugate (Joint Committee on Powder Diffraction Standards). The average crystallite sizes of PMNSs were considered using Debye–Scherrer equation:

$$D = \frac{0.9\lambda}{\beta \cos \theta},$$

where, $D$ is the crystallite size, $\lambda$ is the wavelength of X-ray, $\beta$ is the value of FWHM expressed in radians, and $\theta$ is the Bragg’s angle. Based on this equation, the size of crystallite NPs were determined to be around 12–13 nm. In Lf-PMNSs, the relative diffraction intensity of the magnetic peaks is weaker relative to PMNSs, due to the presence of the amorphous protein on the surface of NPs.

In Fig. 2F, it was found that magnetism saturation reduced steadily with the Lf and Doxo, while, all curves did not show a hysteresis loop. The absence of hysteresis loops in the samples may indicate a superparamagnetic behavior of crystalline particle with average size of smaller than 20 nm. According to our result, NP with superparamagnetic behavior can be easily organized by an external MF. The magnetization saturation of different samples such as PMNSs, Lf-PMNSs and Lf-Doxo-PMNSs was 78, 67, and 65 emu/g, respectively. Besides, the nanosphere structure of the PMNSs was confirmed in the N2 adsorption–desorption method (Fig. 2G). The N2 adsorption–desorption isotherm depicts a kind of IV behavior with an apparent hysteresis loops in the range of 0.45–0.8 and 0.85–1.0 P/P0, representing the nanosphere structure of as-synthesized NPs. The surface area was estimated to be 143.11 m²/g, which is comparatively higher than other reported PMNSs structures.

**Drug loading and release.** The results of Fig. 2H showed that with increasing concentration of drug, despite the constant concentration of PMNSs (0.5 mg/mL), the amount of drug loading in tumor tissue significantly increases on the NP. While, it was shown that the loading efficiency (%) decreases with increasing drug concentrations. This profile suggests that the highest Doxo loading in PMNSs can be achieved in the range of 200–300 μg/mL with an efficiency of over 50%.

The drug release was considered under reservoir-sink with pH of 2.5, 5, and 7.5 at 37 °C. The drug release from Doxo-PMNSs and Lf-Doxo-PMNSs (Fig. 3A) follows a time dependent release profile. Moreover, about 94.3% of drug was released from the Doxo-PMNSs at pH 2.5, whereas 81.3% and 68.9% of drug was released at pH 5.0 and 7.5 after 900 min, respectively. On the other hand, the using Lf as a bio-gate reduced the rate of drug release from PMNSs up to 38%, 46% and 53% in pH 2.5, 5 and 7.5, respectively, compared to Lf-free groups. On the whole, the drug release at pH 2.5 and 5 was much faster and higher than pH 7.5. As shown in Fig. 3A, the initial burst release
at 30 min was 42% in acidic pH and 34% in the neutral pH. This parameter strongly suggests that the pH-sensitive drug release in the case of Doxo-PMNs and Lf-Doxo-PMNs.

**Cytotoxicity assay.** As shown in Fig. 3B, the toxic effects induced by as-synthesized NPs are enhancing by increasing the concentration of free Doxo, PMNs, Lf-PMNs and their combination. Among all groups, Doxo-PMNs and Lf-Doxo-PMNs exhibited the most cytotoxicity against 4T1 cells \((P < 0.05)\). Although, there was no significant difference between PMNs and Lf-Doxo-PMNs at different concentrations, their cytotoxicity was significantly higher than other groups, especially at concentrations of 15 and 20 µg \((P < 0.05)\). In addition, the results show that with increasing concentration of free Doxo compared to PMNs, its cytotoxicity increases. It was also observed that by increasing the concentration of free Doxo, its cytotoxicity would be similar to that of Lf-PMNs. Altogether, the results denoted that the interaction of PMNs and free Doxo can induce a synergistic effect to greatly enhance the 4T1 cell mortality.

**Effect of Doxo and NPs on apoptosis and ROS production.** In order to carry out the Annexin test, different treatments such as PMNs (15 µg/mL), Lf-PMNs (15 µg/mL), Doxo-PMNs (15 µg/mL NPs with 7.5 µg/mL Doxo) and Lf-Doxo-PMNs (15 µg/mL NPs with 7.5 µg/mL Doxo), and 7.5 µg/mL free Doxo were applied and the percentage of apoptotic cells after 24 h was quantified by the flow cytometry (Fig. 4A). The cells incubated with 7.5 µg/mL free Doxo exhibited the induction of apoptosis in Q2 (late apoptotic cells) and Q3 (early apoptotic cells) quadrants in comparison with the control group. The rate of apoptotic cells in these regions increased from %1.85 to %32.3 (17-fold increment, \(*P < 0.001\) and %2.22 to %30.5 (13-fold increment, \(P < 0.001\)) after 24 h, respectively. Similar to the free Doxo, Lf-PMNs induced apoptosis in Q2 (%36.4) and Q3 (%27.8) regions \((P < 0.001)\), while, despite the increased induction of apoptosis by PMNs, the increase in region Q3 (%32.0) was greater than Q2 (%30.2) \((P < 0.05)\) relative to Lf-PMNs. Likewise, it was found that highest reduction in 4T1 cells proliferation and the maximum increase in induction of apoptosis was reached by Doxo-PMNs (26-fold increment) and Lf-Doxo-PMNs (30-fold increment), respectively \((P < 0.001)\).

ROS is generated when cells are under stress, which can be one of the key factors leading to apoptosis. To assay this probability, cells were treated with different therapeutic, i.e., PMNs (15 µg/mL), Lf-PMNs (15 µg/mL), Doxo-PMNs (15 µg/NPs with 7.5 µg/mL Doxo) and Lf-Doxo-PMNs (15 µg/mL NPs with 7.5 µg/mL Doxo), and 7.5 µg/mL free Doxo for 24 h. Our data showed that cells treatment with free Doxo and Lf-PMNs raised the ROS intracellular level up to 189 \((P < 0.01)\) and 191 \((P < 0.01)\) units (Fig. 4C), respectively. On the contrary, despite the remarkable difference between PMNs and control \((P < 0.05)\), ROS generation was meaningfully less than free Doxo and Lf-PMNs. Besides, it can be seen that Doxo- loaded PMNs (2.04-fold) and Lf-PMNs (2.15-fold) produces more ROS compared to control \((P < 0.001)\). Thus, it was determined that Doxo-PMNs and Lf-Doxo-PMNs can stimulate 4T1 cells mortality via production of ROS. Besides, the morphological changes of 4T1 cells treated by different therapeutic such as free Doxo, PMNs, Lf-PMNs, Doxo-PMNs, and Lf-Doxo-PMNs are shown in Fig. 4B. Microscopic images of cells in the presence of Doxo-PMNs and Lf-Doxo-PMNs compared to other groups indicate a cell death of 90% based on apparent morphological changes of cells, including color changes, decrease in cell volume, and deformation.

**Tumor weight.** Figure 5A displays the anti-tumor efficiency of free Doxo, PMNs, Lf-PMNs, Doxo-PMNs, and Lf-Doxo-PMNs in vivo. After injecting 4T1 cancer cells and forming a breast cancer tumor on 18 day, the mice treated with different therapeutics such as free Doxo, PMNs, Lf-PMNs, Doxo-PMNs, and Lf-Doxo-PMNs solutions with a dose of 5 mg Doxo/kg and 10 mg NP/kg (six times with 3 days interval). The treated mice except PMNs group showed smaller RTV on day 18 compared to the control group. However, RTV in the treated groups with Lf-Doxo-PMNs was remarkably less (~3 times) than free Doxo \((P < 0.05)\). Furthermore, at the end of the study, the mean tumors weight in mice treated with Lf-Doxo-PMNs were about 25% and 50% lower than those treated with free Doxo and control groups, respectively (Fig. 5B,C). Overall, the results showed that Lf-Doxo-PMNs in comparison with free Doxo can improve outcome for the treatment of breast cancer and weight loss in vivo.

**Drug bio-distribution.** To evaluate the capability of PMNs with and without Lf on targeted drug delivery, the level of Doxo in vital tissues were determined by HPLC analysis. As shown in Fig. 5D, significant drug accumulation was observed in tumor especially for the Lf-Doxo-PMNs \((P < 0.01)\) 8 h after injection, but with a limited level in kidney, lung, and spleen. In mice that were injected with the Doxo-PMNs and Lf-Doxo-PMNs, drug distribution in the lungs and kidneys was reduced compared to the free Doxo group \((P < 0.05)\), but no significant changes were observed in the liver and spleen. In this line, the fluorescence intensities of Doxo in 5 major organs, tumor and whole-body were shown in Fig. 6A,B. 8 h after injection with free Doxo, Doxo-PMNs and Lf-Doxo-PMNs. The reduction of fluorescence intensity in the kidney, lung, and spleen tissues (Fig. 6B) and its accumulation in the tumor site (Fig. 6A) in the Lf-Doxo-PMNs compared to the control group, indicates a lower accumulation of Doxo in these organs and reducing drug toxicity in off-targeted organs.

Figure 5E shows the distribution of Doxo in BALB/c mice blood at different time intervals of 0.5, 1, 2, 3, 6, 12, 24, 36, and 72 h after injection. As shown in the Fig. 5E, the highest level of Doxo release into the blood at the initial time after injection was observed in the case of free Doxo followed by Doxo-PMNs, and Lf-Doxo-PMNs. However, the highest concentration and lowest blood clearance of drug were belonged to the Lf-Doxo-PMNs and Doxo-PMNs groups within 6 h after injection, respectively. Meanwhile, blood clearance of Doxo was detected much faster in the case of free Doxo group, like the Doxo-PMNs group, than the Lf-Doxo-PMNs.

**Magnetic field and photothermal therapy.** The anti-tumor characteristics of combined chemo-MF therapy, combined chemo-PTT, and combined chemo-MF-PTT based on Lf-Doxo-PMNs were investigated on breast cancer-bearing BALB/c mice based on the change in the tumor size and weight. The results
presented in Fig. 5A show that the mice treated with combined chemo-MF therapy, combined chemo-PTT, and combined chemo-MF-PTT based on Lf-Doxo-PMNSs showed smaller RTV on day 18 compared to the control, free Doxo and even Lf-Doxo-PMNSs groups. Although, there was no significant difference in TV among combined chemo-MF therapy, combined chemo-PTT and combined chemo-MF-PTT therapeutic methods, the results revealed that the RTV in the combined chemo-MF-PTT was markedly reduced in a more significant manner relative to that of the Lf-Doxo-PMNSs group (**P < 0.01). Moreover, the lowest tumor weight at the end of the study was observed in combined chemo-MF-PTT (0.168 g) group followed by combined chemo-PTT (0.234 g), combined chemo-MF therapy (0.274 g) and Lf-Doxo-PMNSs (0.429 g) groups (Fig. 5B). On the whole, the results exhibited that Lf-Doxo-PMNSs-mediated chemotherapy potentially decreases tumor growth in vivo, and the inhibitory effect is increased by combined chemo-MF-PTT.

**Histopathology.** To assess cytotoxicity of the different therapeutic modalities on the major organs, the liver, kidney, lung, and spleen were investigated as targets by the histological assays. As shown in Fig. 6C, histological analysis of tumor tissues displays that the most significant morphological changes in tumor cells are stimulated by combined chemo-MF therapy using Lf-Doxo-PMNSs followed by combined chemo-PTT and combined chemo-MF-PTT. The staining results showed that the structure of tumor tissue was disappeared and some of the cells were shrunken (Fig. 6C, MF, PTT and MF/PTT panels). While, the changes in morphology and cellular...
integrity of tissues treated by different therapeutic, i.e., Lf-PMNSs, free Doxo, and Doxo-PMNSs are relatively less than those of samples exposed to combined chemo-MF-PTT using Lf-Doxo-PMNSs. Commonly, the necrotic or apoptotic cells were stained brown, whereas, the viable cells stained pink. In other side, it was shown that no noticeable tissue damage was found in liver, kidney, and spleen using various therapeutic modalities (Fig. 6C).

Mechanisms of cytotoxicity. To evaluate the mechanisms of apoptosis induced by combined chemo-MF therapy, combined chemo-PTT, and combined chemo-MF-PTT based on Lf-Doxo-PMNSs, expression of some associated genes, such as TNF-α, Bax and Caspase-3, was measured. The genes expression was measured by qPCR technique, and the outcomes are exhibited in Fig. 7. Significant changes in the mRNA expression in tumor cells exposed to combined chemo-MF therapy, combined chemo-PTT, and combined chemo-MF-PTT based on Lf-Doxo-PMNSs indicated that the apoptosis mechanisms were stimulated in different ways. Although chemo-MF therapy, combined chemo-PTT, and combined chemo-MF-PTT can significantly (**P < 0.01) enhance the relative expression of TNF-α, indicating the extrinsic pathway-associated cytotoxicity, intrinsic pathway was significantly increased by combined chemo-MF-PTT. It was also determined that the level of Caspase-3 activity in combined chemo-PTT and combined chemo-MF-PTT groups was higher than other samples. These results indicated that combined Chemo-MF-PTT via Lf-Doxo-PMNSs stimulated apoptosis in the breast cancer cells through activation of the Caspase-3 by initiating both intrinsic and extrinsic apoptotic pathways.
Discussion

PMNSs coated with protein and polymer compounds have received a great deal of interest in the targeted drug delivery due to their ability to be used in combined-modality therapy and imaging capabilities. Herein, we showed the relatively uniform distribution of PMNSs (Fig. 1B) and their available active surface (Fig. 2G), which were in accordance with the study reported by Cao, et al.32, Su, et al.2 and Benyettou, et al.6. While, the ongoing challenges for fabricating these NPs are the limited uniformity between 30 to 300 nm, as well as their low surface area down to 100 m$^2$/g.27,33. On the other hand, SEM and TEM images (Fig. 1A,B), and TGA (Fig. 2D) results displayed that the thickness of coated Lf was like the results of Su, et al.2 and Kanwar, et al.34. The PMNSs can dramatically provide high surface area for the loading of drugs. In this regard, the results of this study (Fig. 2H) and previous studies2,6,27,32 have indicated that the drug loading and its efficiency has been greatly increased due to the high surface area of the as-synthesized PMNSs (~142 m$^2$/g). Also, the cytotoxic effects of PMNSs, Lf-Doxo-PMNSs and other groups against 4T1 breast cancer cells show the dose-dependent cells growth inhibition induced by NPs as exhibited in the Fig. 3B. The cytotoxicity of IONSs is generally expressed by catalytic effects through the ROS production, changes in intracellular biological proteins, damage to DNA, and disruption of electron transport in mitochondria.8,35. These results are consistent with the finding of Su, et al.2, Kanwar, et al.34 and Gholami, et al.31 which shows Lf-Doxo-PMNSs induced a more promising inhibitory effect on the proliferation of cancerous cells compared to other treated groups. It is likely that the more inhibitory effect of Lf-Doxo-PMNSs in comparison with other groups is due to their greater penetration into cells based on reducing drug resistance in cancer cells and toxic effects on cellular activities.2 Therefore, this case can play a potential role
in apoptosis induction of cell. In this regard, as revealed by Kanwar, et al.\textsuperscript{34}, Fig. 4A,C show that Lf-Doxo-PMNSs can stimulate apoptosis and production of ROS, respectively.

Figures 5D and 6A revealed that the injection of free Doxo in blood, in addition to accumulation in the tumor, causes its accumulation in all major organs, especially the liver, kidney and spleen. Whereas, by using Doxo-PMNSs, it was found that the level of Doxo accumulation, in agreement with the study of Nigam and Bahadur\textsuperscript{36}, is significantly reduced especially in the kidney, lung and spleen. This accumulation can have a direct relation with the accumulation of PMNSs in the main organs based on the potential activity of macrophages\textsuperscript{37}. Hence, applying Lf as a coating on Doxo-PMNSs is expected to significantly augment the Doxo-equivalent doses in the tumor based on reducing drug resistance. In this regard, Kanwar, \emph{et al.}\textsuperscript{34} demonstrated that the use of Lf as coatings not only reduces the accumulation of drugs in the main organs, but also increases the drug performance by increasing the drug level in the tumor tissue. This incident suggests that the use of Lf leads to targeted Doxo distribution in the body toward tumor site. Moreover, according to Fig. 5E, by applying Lf coating on the Doxo-PMNSs, the rapid blood clearance of the Doxo decreases, due to the nature of the protein corona formed on the Lf-Doxo-PMNSs as well as the escape from the MPS\textsuperscript{38}. Also, contrary to the results of Kanwar, \emph{et al.}\textsuperscript{34}, Fig. 3A displays that the use of Lf in Doxo-PMNSs regulates the release rate of the Doxo under various pH conditions. Thus, Lf serves as an effective acid-susceptible bio-gate in controlling the initial–burst release of Doxo and increases the drug release in tumor tissue with acidic environment. On the other hand, in agreement with Benyettou, \emph{et al.}\textsuperscript{6} and Gholami, \emph{et al.}\textsuperscript{31}, Fig. 3A reveals that Doxo-PMNSs have the highest rate of drug release in acidic environment. The main mechanism of drug release is related to the effect of the endosomes, lysosomes and acidic pH in tumor cells on the binding affinity of PMNSs/Doxo\textsuperscript{39}. The findings of Fig. 2F also show that the use of Lf and Doxo did not have an adverse effect on the magnetic properties of the PMNSs. Therefore, the Lf-Doxo-PMNSs, similar to that of Su, \emph{et al.}\textsuperscript{2} and Kanwar, \emph{et al.}\textsuperscript{34}, can be useful in MF-based drug delivery, optical therapies, and imaging activities.

In another part of this study, the effects of Lf-Doxo-PMNSs and combined chemo-MF-PTT were investigated. The data in Fig. 5A,B reveals that Lf-Doxo-PMNSs and Doxo-PMNSs, respectively, significantly decrease the RTV and tumour weight of the breast cancer. Our results are similar to the anti-tumour activities of different nano-based platforms of Doxo\textsuperscript{40}. The most anti-cancer efficacy of Lf-Doxo-PMNSs among all NPs could
be illustrated by raising the level of Doxo in the tumour site, which can be detected by prolonged blood circulation time of drug (Fig. 5E) and its potential accumulation in the tumor (Fig. 6A,B). Despite the positive effects of Lf-Doxo-PMNSs on reducing the RTV and tumor weight, as shown in Fig. 5A,B, the use of auxiliary treatments including combined Doxo-MF therapy, combined chemo-PTT, and combined chemo-MF-PTT can exacerbate the inhibitory effects of Lf-Doxo-PMNSs on the breast cancer tumor. With the introduction of MF and PTT, the inhibitory effect of Lf-Doxo-PMNSs was enhanced by stimulating the extrinsic pathway of apoptosis through overexpression of the TNF-α mRNA (Fig. 7). Our findings are similar to the results of Espinosa, et al.\(^1\) and Li, et al.\(^3\) demonstrated that the application of combined Lf-Doxo-PMNSs-MF therapy and Lf-Doxo-PMNSs-PTT can induce the extrinsic pathway of apoptosis due to the inflammation stimulated by heat (up to 50 °C). While, inhibiting tumor growth by combined Lf-Doxo-PMNSs-MF-PTT occurs through enhancing Bax mRNA expression (Fig. 7) via initiating intrinsic pathway of apoptosis. Since, IONS inhibits tumor growth by enhancing ROS\(^4,44\), it is assumed that the reduction of tumor growth in the presence of Lf-Doxo-PMNSs induced by different combined-modality therapy was done through ROS production, which provide an essential role in the regulation of apoptosis, especially in tumor cells. In addition, (Fig. 7), the results of this study indicate that combined-modality therapy can stimulate the activation of Caspase-3 with different mechanisms. In this regard, Zanganeh, et al.\(^5\) using IONS and increasing the activity of Caspase-3, were able to prevent the development of liver cancer. Also, histological assays showed that the Lf-Doxo-PMNSs has the lowest cytotoxicity on major organ cells compared to other free Doxo and Doxo-PMNSs.\(^42\)

**Conclusions**

In this research, we developed a scalable procedure for the synthesis of highly uniformed porous PMNSs coated with Lf. PMNSs were capable of encapsulating Doxo with high efficiency for potential targeted drug delivery to breast tumors based on Lf coatings and pH-sensitive drug release. The results showed that Lf-Doxo-PMNSs can prolong the circulation time of Doxo in the blood as well as a targeted drug delivery and reducing drug resistance in the cancer tissue. The outcomes from *in vitro* and *in vivo* trials showed that Lf-Doxo-PMNSs stimulated the apoptosis to reduce TV with the lowest cytotoxicity on the normal tissues. In this line, the histological results confirmed that the combined-modality therapy by Lf-Doxo-PMNSs could result in significant morphological changes of breast cancer cells, whereas the minimum cytotoxicity was observed in the main tissues. It was also revealed that the combined chemo-MF therapy, combined chemo-PTT, and combined chemo-MF-PTT - based on Lf-Doxo-PMNSs could significantly reduce the volume and size of breast tumor, by inducing extrinsic (TNF-α) and intrinsic (Bax) pathways of the apoptosis. Overall, we believe that targeting drug delivery and reducing drug resistance of cancers cells based on Lf-Doxo-PMNSs, along with integration of therapeutic modalities can enhance the effectiveness of the multi-modality approaches in breast cancer therapy.

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Author contributions

M.S., M.F., A.H.; Designed and performed experiments, analysed data and co-wrote the paper. E.N., H.Y.-M.; Performed experiments. N.M.Q., A.S., F.M.A.; Performed analyses. M.S.S., K.M.A.; Performed collected sample and transporter experiments. F.A.Q., H.A.M.; Performed metabolite analyses; M.F.; Supervised the research.

Competing interests

The authors declare no competing interests.
