Multifunctional PD-L1 Targeted $\text{Fe}_3\text{O}_4@DOX$ Nanoparticles Under MRI Monitoring For Combination Therapy of Triple-Negative Breast Cancer

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Abstract

Background: The triple negative breast cancer (TNBC) is the most difficult subtype of breast cancer to treat, with currently restricted treatment of chemotherapy. However, evidence suggests that immunotherapy is only effective for a small portion of patients. The combination therapy of both immunotherapy and chemotherapy has demonstrated great promise in its treatment efficiency. However, this combination therapy is limited by both the organ toxicity of the chemotherapeutic drugs and the inaccessibility of the in vivo monitoring of individual tumor response to therapies. With the development of the nanomedicine, synchronous targeting therapy, imaging diagnosis and monitoring can be achieved conveniently using the multifunctional nano-platform for TNBC.

Results: In our study, we prepared the DOX loaded Fe_3O_4 nanoparticles targeting the PD-L1 (PD-L1@ Fe_3O_4 -DOX, FPD) to obtain the real-time magnetic resonance imaging (MRI) monitoring and combination therapy of both chemotherapy and immunotherapy for TNBC. The results showed that FPD inhibited tumor growth more effectively than either DOX chemotherapy or PD-L1 immunotherapy alone.

Conclusion: Our study demonstrated that FPD has shown a great potential for theranostics and clinical translation in synchronous MRI imaging-guided monitoring and combined treatment of both DOX and immunotherapy of TNBC.

Introduction

Breast cancer is a severe challenge in women health. The incidence rate of breast cancer has generally been rising over the past 50 years with rapid increases observed particularly in developing countries [1, 2]. Different subtypes of breast cancers arise from different gene mutations occurring, causing difficulty in breast cancer diagnosis and treatment [3]. Among these subtypes, triple-negative breast cancer (TNBC) is highly heterogeneous and is one of the most severe forms of breast cancer [4].

Surgical removal is generally the main treatment for early breast cancer. However, compared with other subtypes, TNBC patients have shown higher risk of postoperative recurrence and distal metastasis, and lower survival rate. Furthermore, due to the lack of the expression of progesterone receptor (PR), estrogen receptor (ER), and human epidermal growth factor receptor (HER2) in TNBC, current molecular targeted therapy and hormone therapy are ineffective in treating TNBC patients [5]. Therefore, the traditional chemotherapy remains the primary treatment option for patients with TNBC at intermediate and advanced levels [6].

Doxorubicin (DOX) is used in many chemotherapy regimens due to its prominent and broad-spectrum antineoplastic characteristics [7]. The main mechanism of action of DOX is that the product embeds into DNA and inhibits the synthesis of nucleic acid, showing the strong cytotoxic effect. As a type of periodic non-specific drug, DOX shows a wide anti-tumor spectrum and is commonly used in the treatment of breast cancer [8]. However, the application of DOX has been hindered by toxicities such as hematopoietic suppression, nausea, extravasation, and alopecia, with cardiotoxicity as the most severe side-effect,

leading to cardiomyopathy and/or congestive heart failure. Till now, there still exists an urgent medical demand to explore pharmacotherapeutic strategies and reduces the side effects, that can improve the treatment outcome of TNBC [9].

Immunotherapy provides a new option and direction for TNBC treatment. The host immune responses to tumor cells can be induced by tumor-infiltrating lymphocytes (TILs) present within the tumor microenvironment, while the TNBC is more likely to harbor TILs than other subtypes of breast cancer. Tumor cells can overexpress immune checkpoint-related molecules to inhibit T cell activity and escape the body's immune response to survive [10]. The immune checkpoint inhibitors (ICBs) can inhibit the activity of immune checkpoint molecules to release the activity of T cells and restore the killing effect on tumor cells to ultimately achieve the anti-tumor effect [11]. At present, there are mainly two types of ICBs, including CTLA-4 receptor inhibitors and PD-1/PD-L1 inhibitors [12]. However, a large number of studies have shown that the single drug treatment of immune checkpoint inhibitors has limited benefits for TNBC patients with the objective response rate (ORR) ranging from 5% in PD-1/PD-L1 positive population to 23% in first-line PD-L1 positive population [13].

Studies have shown that several chemotherapeutic agents commonly used in breast cancer treatments can promote immunogenic cell death, thereby releasing tumor cell antigens and promoting immune response [14]. Therefore, chemotherapeutic agents combined with immune checkpoint inhibitors may achieve enhanced therapeutic effects [15]. However, this combination therapy is limited by the organ toxicity of the chemotherapeutic drugs and the inaccessibility of in vivo monitoring of individual tumor response to therapies. Therefore, the current focus of TNBC treatment is to improve the therapeutic effect and to reduce drug toxicity and side effects. It has been suggested that effective combination therapy and improvement on the targeting of drugs for diagnosis and treatment are the main potential solutions to these problems [16, 17].

Furthermore, various imaging techniques, including infrared scanning, mammography, computed tomography (CT), magnetic resonance imaging (MRI), ultrasound, and positron emission tomography (PET), may provide the diagnosis, assessment, and monitoring for breast cancer [18]. Among these imaging technologies, MRI has become one of the most important screening methods for breast diseases due to its significant resolution on soft tissues and high detection sensitivity (~ 96%), which can reduce the number of false positives in mammography examination and avoid unnecessary biopsy. However, the limitation of MRI in the actual clinical practice is that MRI is not capable of achieving targeted imaging of lesions and real-time monitoring of therapeutic drugs [19–21].

In recent years, with the rapid development of nanomedicine, multifunctional nanosystems with multiple diagnostic and therapeutic effects have shown significant potential in the real-time targeted and accurate diagnosis and treatment of malignant tumors, providing the potential solutions to the problems of TNBC [22]. Studies have demonstrated that as an ideal targeted contrast agent for MRI, the iron oxide nanoparticles ($\text{Fe}_3\text{O}_4\text{-NP}_s$) have shown higher biological safety, relaxation, and adjustable surface modification than both the manganese oxide nanoparticles and clinical chelated gadolinium contrast

agents. Furthermore, due to its sound magnetic characteristics and biocompatibility, the surface of Fe_3O_4 -NP_S is easy to be modified, making Fe_3O_4 -NP_S the suitable materials to construct targeted drug delivery carrier system [23, 24].

In our study, we developed and characterized a MRI-based Fe_3O_4 -NP_S with targeted PD-L1 antibody and linked DOX (PD-L1@Fe₃O₄-DOX, FPD). This nanohybrid technology aims to establish and enhance the synchronous MRI monitoring and combined chemotherapy and immunotherapy for TNBC, ultimately to improve the therapy efficacy in the treatment of TNBC.

Materials And Methods

Chemicals

The $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, oleic acid, sodium oleate, sodium carbonate, octadecene, 2,3-dimercaptosuccinic acid (DMSA). The n-hexane, tetrahydrofuran, and ethanol were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and sulfo-N-hydroxysuccinimide (S-NHS) were purchased from the Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Mouse anti-PD-L1 antibody was purchased from Bioxccl (West Lebanon, NH, USA), and DOX from MCE (Shanghai, China).

Synthesis and modification of Fe_3O_4 nanoparticles

The Fe_3O_4 nanoparticles were synthesized based on a previous study with some modification [25]. Briefly, 10.8 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 36.5 g of sodium oleate were dissolved in a mixed solvent composed of 60 mL distilled water, 80 mL ethanol, and 140 mL hexane. The mixed solution was stirred and heated to 70°C and kept for 4 h. The upper organic layer was then collected, washed with distilled water, and dried by evaporation to obtain the waxy material, i.e., the iron-oleate complex. The iron-oleate complex was mixed well with 5.7 g oleic acid and 200 g of 1-octadecene, then heated to 320°C and kept for 30 min. The resulting mixture was cooled to room temperature and added with an excessive amount of ethanol to precipitate the nanoparticles. The solution was centrifuged in an Eppendorf Centrifuge (Model: 5804/5804 R, USA) to collect the nanoparticles of Fe_3O_4 .

The Fe_3O_4 nanoparticles were then modified with DMSA molecule to achieve the high water dispersity. Briefly, 30 mg Fe_3O_4 nanoparticles and 40 mg DMSA were added to the solution of Na_2CO_3 , and then 2 mL tetrahydrofuran was added to the mixture. The reaction solution was mixed via ultrasonication for 30 min and shaken for 2 h. The DMSA modified Fe_3O_4 nanoparticles were obtained by freeze-drying.

Conjugation of PD-L1 and DOX on the surface of Fe_3O_4 nanoparticles

The Fe_3O_4 -PD-L1/DOX was synthesized through the conjugation of PD-L1 antibody and DOX molecule on the surface of Fe_3O_4 -DMSA nanoparticles. The solution of Fe_3O_4 -DMSA nanoparticles (2 mg/mL) added with 2 mM EDC and 5 mM S-NHS were kept at room temperature for 30 min. Then, 100 μg DOX and 50 μL PD-L1 antibody were added to the mixture and kept at 4°C overnight. The obtained Fe_3O_4 -PD-L1/DOX complex (FPD) was purified through dialysis. Both the Fe_3O_4 -DOX (FD) and Fe_3O_4 -PD-L1 (FP) were obtained through the same procedures as described.

Material characterization

The transmission electron microscopy (TEM; JEM-2100, JEOL, Tokyo, Japan) and high-resolution transmission electron microscopy (HRTEM, JEM-2100, JEOL, Tokyo, Japan) were used to obtain the morphological and crystal structural characteristics of the Fe_3O_4 -related nanoparticles, respectively. X-ray diffraction analysis (XRD) was performed on a Bruker D8 advanced powder diffractometer with a Cu K α X-ray source (D8 Advance, Bruker, Germany) to characterize the structure of Fe_3O_4 nanoparticles. Fourier transform infrared (FTIR) spectra were obtained with an infrared spectrophotometer (Nicolet Nexus 670, Thermo Fisher Scientific, Inc., Walsham, MA, USA) to analyze the surface functional groups of the nanomaterials. The saturation magnetization of the Fe_3O_4 nanoparticles was obtained on the vibrating sample magnetometer (MicroMagTM Model 2900 AGM system, Princeton Measurements Corp., Westerville, OH, USA). The ultraviolet – visible (UV – vis) light absorption spectra of the synthesized aqueous were analyzed from an UV – vis spectrophotometer (UV-6100, Meipuda, Xi'an, China). The surface charge properties and size distribution of Fe_3O_4 -related nanoparticles in their aqueous suspension were characterized by the Zeta Potential Analyzer (ZetaPALS, Brookhaven Instrument Corp, Holtsville, NY, USA). The relaxation rate of MRI images of Fe_3O_4 was analyzed on a 3.0 T GE Signa HDx scanner (GE Healthcare, Milwaukee, Wisconsin, USA).

Cell culture and mouse model of TNBC

The mouse TNBC cells 4T1 line and human normal mammary cell MCF-10A were purchased from the Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd. (Shanghai, China). All cells were cultured in RPMI medium 1640 (Gibco, Shanghai, China) with 10% fetal bovine serum (Gibco, USA) at 37°C in 5% CO_2 .

Male BALB/c mice (4 weeks old) were purchased from the Animal Center at Shandong University. These mice were 4T1 injected with 1×10^7 4T1 cells in their right mammary glands. The mice were treated when the tumors grew to the approximately 100 mm^3 . Experiments were approved by the Ethics Committee of Qilu Hospital of Shandong University and conducted according to institutional guidelines for animal care.

Immunofluorescence

In order to evaluate the feasibility of the target, 4T1 cells and tumor tissue sections were stained with immunofluorescence as previously described [26]. The antibodies used are as follows: anti-PD-L1 antibody (1:500; MCE) and Alexa Fluor 488 antibody (1:2,000; MCE). The cells or tissue sections were

incubated with DAPI (1:1000; Sigma-Aldrich) for 5 min. Fluorescence imaging with confocal microscopy (Zeiss LSM800, Germany).

Cytotoxicity of nanomaterials

The cytotoxicity of Fe₃O₄ nanospheres on cell viability was evaluated by the Cell Counting Kit-8 (CCK-8) assay (Beyotime, Jiangsu, China). The 4T1 and MCF-10A cells were seeded on the 96-well plates at a density of 8.0×10^3 cells/well to complete the adhesion of 24 h. The cells were added with various concentrations of Fe₃O₄ (25, 50, 100, and 200 µg/ml) and were incubated for 6, 12, 24, and 48 h, respectively. The control cells were cultured in the regular medium without any stimuli. A total of 10 µl of CCK8 solution was added to each well, and the plates were incubated at 37°C for 2 h. Absorbance was measured at 450 nm (Molecular Devices, VT, USA). Cell viability of control cells was considered 100%.

Cellular uptake of nanoparticles

The cellular uptake of nanoparticles was evaluated by prussian blue staining, which was used to detect the presence of iron element, following the procedures reported previously [27]. The cells were observed under a light microscope to determine the intracellular distribution of nanoparticles.

To further verify *in vitro* the targeting and cellular uptake of the nanoparticles, the concentration of Fe was measured by inductively coupled plasma-mass spectrometry (ICP-MS; Agilent Technologies, UK) following the procedures reported previously [28]. The number of Fe nanoparticles in a single cell was calculated by the equation: $n = (N/D)/M$, where N was the total number of atoms, D was the cell density in the ICP-MS sample, and M was the mass of a single Fe nanoparticle.

Fe₃O₄, FP and FPD were incubated in a humidified incubator (37 °C, 5% CO₂) for 24 hours, and the uptake of nanoparticles was observed by TEM (Model: TEM-1200, Tokyo, Japan).

To evaluate the imaging efficiency of nanoparticles *in vitro*, vials were prepared containing 4T1 cells (5×10^6 cells) with 200 µL nanoparticles (Fe₃O₄ and FP) added, incubated for 2 h (37°C and 5% CO₂). Following incubation, cells were collected with a scraper, washed with PBS, and centrifuged at 500 rpm, with the pellet resuspended in PBS for MRI study. The nanocomposite was tested for MRI signal intensity using 3.0 T MRI clinical instrument (GE Medical Systems, software version HD 16.0_V03_181638.a).

DOX efficacy in vitro

Double staining with both Hoechst 33342 and Propidium Iodide (PI) (Sigma Aldrich, St. Louis, USA) was used to investigate the cell apoptosis, and the experimental operation was carried out according to the manufacturer's instructions. This study determines the proportion of viable cells (PI negative/Hoechst 33342 positive),

To further quantitatively evaluate the local effect of DOX, ROS staining was performed using Dichlorodihydrofluorescein diacetate assay (DCFH-DA) following the manufacturer's protocol. After each

group was cultured based on the treatment methods described above, the cells were washed thrice with PBS and incubated in DCFH-DA at 37°C for 20 min. The cells were observed under a fluorescence microscope. The percentage of ROS-positive cells were calculated by dividing the total number of ROS-positive cells by the total number of cells in the same field.

Material relaxation rate and in vivo MRI imaging

MRI were performed using a clinical 3.0-T MRI scanner (GE company Discovery MR750) with an 8 circular head coil at room temperature. Fast spin echo (FSE) T2-weighted images (T2WI) and T2-mapping were acquired, and T2- mapping was also acquired using single section multi-spin-echo sequences. The detailed acquisition parameters of T2-weighted images were as follows: TR/TE = 5000/111 ms, FOV = 100 mm, matrix of 256×256, section thickness of 2 mm, a circular region of interest was selected in each sample, and the values of T2 relaxation times were obtained. The T2 relaxivities of FPD were calculated from the slope of the linear plots of the r_2 relaxation rates ($1/T_2$) versus iron concentration. The increase in the r_2 relaxation rates ($1/T_2$) with increasing iron concentration was analyzed by the linear least squares regression analysis. At least four mice per group were included in this analysis. The carrier-to-noise ratio (CNR) in tumor was calculated as follows: $CNR = [Tumor\ SNR\ (Signal\text{-}to\text{-}noise\ ratio) - Muscle\ SNR] / (standard\ deviation\ of\ background)$.

Efficacy of FPD in vivo

The mice with 4T1 tumor were divided into 5 treatment groups ($n = 5$): (1) PBS (treated with PBS), (2) Fe_3O_4 (treated with Fe_3O_4), (3) FP (treated with FP), (4) FD (treated with FD), and (5) FPD (treated with FPD). When the tumor size reached about 100 mm, different groups of nanoparticles was injected into the mice through the tail vein, while the control animals received the equivalent volume of sterile PBS. Starting from Day 0, the mice were intravenously injected with different groups of nanoparticles or PBS and the tumor diameters were measured by a digital caliper every three days for a total of 15 days. The tumor volume in mm^3 was calculated using the following formula: $Volume = 0.5 \times a \times b^2$, where a and b were the largest and the smallest diameters, respectively. The body weight of mice was measured every 3 days with the survival of the mice also determined.

Tissue preparation

The mice were anesthetized and killed 15 days after the experiments with the tumors removed, weighed, and sectioned. Tumor tissue and major organs were removed and fixed in paraformaldehyde for over 2 days, then embedded in paraffin and sectioned at a thickness of 3 μm .

Hematoxylin and Eosin (HE) staining

The morphological characteristics of tumor tissue and major organs were observed based on HE staining of the paraffin sections, which were dewaxed and dehydrated with a gradient of alcohol of a series of

concentrations. The HE staining was performed using an HE staining kit (Beyotime, Jiangsu, China) according to the manufacturer's instructions. The sections were observed under a microscope (Olympus, BX60, Tokyo)

Tunel staining

Sections of tumor tissue and major organs were incubated with proteinase K solution for 15 min followed by incubation with TUNEL reaction mixture (Beyotime, Beijing, China). After washing thrice with PBS, the sections were mounted with a mounting solution containing the nuclear dye DAPI and observed using either a conventional fluorescence microscope or a confocal laser fluorescence microscope.

qRT-PCR analysis

Quantitative real-time PCR was performed to assess the expression levels of genes *Bcl-2*, *Bax*, and *caspase-3*. Primer sets used were as follows: GAPDH (F) 5'-GTATGACTCCACTCACGG-3' and (R) 5'-GGTCTGGCTCCTGGAAGA-3', Bax (F) 5'-TCAGGATGCGTCCACCAAGAAG-3' and (R) 5'-TGTGTCCACGGCGGCAATCATC-3', Bcl-2 (F) 5'-ATCGCCCTGTGGA TGA CTGAGT-3' and (R) 5'-GCCAGGAGAAATCAAACAGAGGC-3', and Caspase-3 (F) 5'-GGAAGCGAATCAATGGACTCTGG - 3' and (R) 5'-GCATCGACATCTGTACCAGACC-3'. Total RNA was extracted from the tumor tissue and major organs after corresponding treatments by using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. The RNA (1 µg) of each sample was reversely transcribed into cDNA using a Kit (Takara, Japan) and PCR-amplified using the TaKaRa Taq Kit (Takara, Japan).

Flow cytometry

The number of CD3⁺CD8⁺ T cells in the tumor was studied by flow cytometry based on the gating strategies as reported previously [29]. The T cells were labeled with both anti-CD3 (Abcam, ab16669) and anti-CD8 antibodies (Abcam, ab203035).

Statistical analysis

All data were analyzed with SPSS (version 17.0; SPSS, USA). The data were presented as the mean ± standard error of the mean (SE) with the statistical significance ($p < 0.05$) determined using Student's t-test.

Results

Characterization of nanomaterials

The morphological characteristics and crystal structural features of synthesized nanoparticles were investigated by TEM and HRTEM, respectively. As shown in Fig. 2A, the nanoparticles were of uniform shape with the size of ~ 20 nm. The HRTEM image (Fig. 1B) of the obtained nanoparticles showed that the nanoparticles were of high crystallinity with the crystal plane spacing ($d = 0.29$ nm) corresponding to the (422) crystal face. After the modification by the DMSA molecule, the Fe₃O₄-DMSA showed high

monodispersity in water (Fig. S1B) due to the abundant carboxyl group on the surface of Fe_3O_4 nanoparticles. The morphological observations of the Fe_3O_4 nanoparticles after the conjugation with DOX molecule, PD-L1 antibody, and both DOX molecule and PD-L1 antibody on the surface of Fe_3O_4 -DMSA nanoparticles were shown in Fig. S1C, Fig. S1D, and Fig. 1C, respectively. The conjugation of DOX molecule showed no evident effect on the morphology of the Fe_3O_4 nanoparticles (Fig. S1C). However, after the conjugation of PD-L1 antibody, the surface of Fe_3O_4 was coated by a layer of organic component, probably due to the PD-L1 antibody with high molecular weight (Fig. S1D). Similarly, after the conjugation of both DOX molecule and PD-L1 antibody, the Fe_3O_4 nanoparticles were also coated by an organic layer (Fig. 1C). The XRD pattern showed that the synthesized nanoparticles corresponded to the standard PDF card (19-0629) (Fig. 1D; Fig. S1A), indicating that the nanoparticles were the pure Fe_3O_4 phase with high crystallinity. These results were consistent with those of the HRTEM observations. The superparamagnetic property of the synthesized Fe_3O_4 nanoparticles was measured through the saturation magnetization curve based on a vibrating sample magnetometer with the saturation magnetization at room temperature confirmed as large as 53 emu/g (Fig. 1E). To confirm the modification of DMSA molecule on the surface of Fe_3O_4 nanoparticles, the FTIR spectra were analyzed (Fig. 1F). For the sample of Fe_3O_4 , the strong peaks located at 2916 and 2847 cm^{-1} corresponded to the C-H stretching vibration of methylene due to the remained oleic acid molecule on the surface of Fe_3O_4 nanoparticles, while the broad peak around 2037 cm^{-1} corresponded to C-O stretching vibration. For the samples of Fe_3O_4 -DMSA, the sharp peaks at 1569 and 1360 cm^{-1} corresponded to the abundant symmetric and asymmetric stretches of carboxylate (COO^-), while the broad peak at 3243 cm^{-1} were caused by the O-H stretching vibration. The peaks located at 1046 cm^{-1} corresponded to the C-O stretching vibration. These peaks indicated the abundance of DMSA molecules. The FTIR results confirmed that the DMSA molecules were successfully modified on the surface of Fe_3O_4 nanoparticles. The UV-Vis absorbance spectrum of Fe_3O_4 -DMSA, Fe_3O_4 -DOX, Fe_3O_4 -PD-L1, and Fe_3O_4 -PD-L1/DOX were shown in Fig. 1G. The results indicated that the conjugation of PD-L1 antibody showed no effect on the absorbance spectrum, whereas the conjugation of DOX molecule largely increased the absorption from the wavelength of 600 nm to 1000 nm, strongly confirming the conjugation of DOX molecule on the surface of Fe_3O_4 nanoparticles. The zeta potential values of Fe_3O_4 -DMSA, Fe_3O_4 -DOX, Fe_3O_4 -PD-L1, and Fe_3O_4 -PD-L1/DOX were -59.4 ± 6.6 mV, -42 ± 10.2 mV, -28.7 ± 4.63 mV, and -38.9 ± 7.18 mV, respectively (Fig. 1H). The large negative value of Fe_3O_4 -DMSA aqueous was due to the abundant carboxyl group of DMSA molecule on the surface of Fe_3O_4 nanoparticles, attributing to the high monodispersity of the aqueous. After the conjugation of PD-L1 antibody and DOX, the zeta potential increased, probably due to the consumption of carboxyl group on the surface of Fe_3O_4 nanoparticles. The size distribution of these samples were investigated. Results showed that the Z-Average sizes of Fe_3O_4 -DMSA, Fe_3O_4 -DOX, Fe_3O_4 -PD-L1, and Fe_3O_4 -PD-L1/DOX were 35.7 nm, 61.6 nm, 182.9 nm, and 237.7 nm, respectively (Fig. 1I). These results were consistent with those based on TEM observations.

In vitro imaging

We investigated the feasibility of imaging protein targets PD-L1 in 4T1 cells (Fig. 2A). The results showed that PD-L1 was widely expressed on the surface of 4T1 cells, confirming its feasibility as a target.

To further verify the targeting of nanoparticles and the effectiveness of MRI in 4T1 cells, post-uptake MRI was performed on non-targeted (Fe_3O_4) and targeted (FP) groups of nanoparticles (Fig. 2B). The results showed that T2WI of the targeted group was significantly lower than that of the non-targeted group, and the signal intensity in the targeted group decreased with the increase of concentration, indicating that the uptake of nanoparticles in the targeted group was more than that in the non-targeted group ($P < 0.001$) in a concentration-dependent manner (Fig. 2C).

To see the in vitro uptake properties of these nanoparticles, we carried out Prussian blue staining and iron assay on 4T1 cells (Fig. 2D, E). Under the same concentrations (10–40 $\mu\text{g}/\text{mL}$), FPD was stained stronger than Fe_3O_4 group. ICP-MS was performed to detect the iron content (Fig. 2E). Compared with Fe_3O_4 group, the FPD group showed significantly higher uptake for iron content ($P < 0.001$).

The cellular uptake of nanoparticles and ultrastructural features in 4T1 cells were observed using TEM (Fig. 2F). More FP/FPD nanoparticles were internalized compared to Fe_3O_4 nanoparticles with the nanoparticles gradually peaked at 4 h and dissipated at 24 h.

Therapeutic efficacy of nanoparticles in vitro

The CCK-8 method was used to investigate the toxicity of Fe_3O_4 nanoparticles to cells. In 6, 12, 24, and 48 h, these nanoparticles did not show noticeable cytotoxicity toward 4T1 and MCF-10A cells (Fig. 3A).

Double staining of Hoechst 33342 and PI was used to evaluate cell apoptosis (Fig. 4B). The results showed that the nuclei of apoptotic cells in FD and FPD groups emitting a shrunken, fragmented with strong blue and red fluorescence. The results of the quantitative assessment showed that the survival rates of normal cells in FPD group were lower than those in FD group (Fig. 3C; $P < 0.05$).

The ROS level in the FD/FPD group was significantly higher ($P < 0.05$) than that in the Fe_3O_4 /FP group, indicating that DOX led to elevated ROS levels in the cells, while the ROS level of FPD group was significantly higher than that of FD group (Fig. 3D, E).

MRI imaging in vivo

We measured the relaxation rate of FPD to evaluate the performance of FPD as a contrast agent in MRI. T_2 WI images and pseudo color map of FPD at 0.025325, 0.05065, 0.1013, 0.2026, and 0.4052 mM were shown in Fig. 5A. The fitting curve (Fig. 4B) obtained showed that the relaxation rate of FPD was $146.43 \text{ mM}^{-1} \text{ s}^{-1}$, which is far high the relaxivities of usual MRI contrast agents, indicating that FPD can be used as a negative contrast agent for T_2 WI.

Next, T2-weighted MRI scans were acquired before and after the intravenous injection of FPD at different time points (1, 4, 8, and 24 h) for the mice with 4T1 tumor. As shown in Fig. 4C-D, MRI showed that the

lowest signal value was achieved in the tumor site at 4 h after probe injection both in Fe_3O_4 and FPD group, but a more significant decreased MRI signal value in the tumor region was detected in FPD group ($P < 0.001$). And the signal value at tumor site gradually recovered to its initial state 24 hours after nanoparticles injection. These results were consistent with the results of cell imaging, supporting the specific targeting of FPD to tumors. FPD aggregates much more in tumor sites than Fe_3O_4 , suggesting that FPD can be used as an optimal probe for *in vivo* MRI to improve the accuracy of biomedical imaging.

Efficacy of combination therapy in vivo

To determine the synergistic benefit of DOX combined with PD-L1 immunotherapy, we performed the *in vivo* antitumor experiments.

The mice with 4T1 tumor were divided into 5 groups and treated with PBS, Fe_3O_4 , FP, FD, or FPD, respectively. The body weight and tumor volumes in mice were continuously monitored to assess the antitumor efficacy for a total of 15 days (Fig. 5A). No significant differences in tumor volume were observed between the PBS and the Fe_3O_4 groups (Fig. 5B, C). The volume of tumor in FD group were significantly smaller than that in the FP group, while the FPD group showed the smallest tumor volume, indicating the highest antitumor efficacy. The survival rate of mice with different treatments was monitored to the end of the experiment. The data suggested that the treatments of DOX and PD-L1 group increased the survival rate compared to other two groups, the increased potential tumor therapeutic effects due to the combination therapy of DOX and PD-L1 immunotherapy (Fig. 5D). Besides, the mouse body weight had no significant difference among the five groups of mice until the end of the trial (Fig. 5E).

Efficacy of in vivo chemotherapy

To further verify the therapeutic effect of the nanomaterials on tumor tissue, the TUNEL apoptotic assay was performed on the tumor tissues (Fig. 6A, B). Results showed that the therapeutic effect of FPD on tumor tissues was significantly increased in comparison to that of other groups ($P < 0.01$). Furthermore, in order to further verify the biosafety of the nanomaterials, we also performed TUNEL staining on the major organs of the mice to mark apoptotic cells. Results showed that few apoptotic cells were observed on the major organs, including the heart, liver, spleen, lung, and kidney (Fig. 6C, D). The qRT-PCR results of three apoptosis-associated genes (Caspase-3, bax and bcl-2) revealed that FPD was not only lethal to the tumor tissue, but also safe compared with the control group (Fig. 6E). Moreover, the results of HE staining also revealed that the nanoparticles showed low organotoxicity (Fig. S2).

Efficacy of in vivo immunotherapy

We performed immunofluorescence on PD-L1 in tumor tissues to further verify the feasibility of PD-L1 as a target of the nanoparticles (Fig. 7A). The results of Prussian blue staining on tumor tissue sections showed that the tumor absorption capability in the FPD group was significantly higher than that in the control group (Fig. 7B).

In order to verify the efficacy of immunotherapy in vivo, the contents of CD3⁺CD8⁺ T cells in tumor tissues were measured. Flow cytometry test results showed that the content of CD3⁺CD8⁺ T cells in the FPD group was significantly higher than that in the control group and FD group (Fig. 7C, D), while there were more CD3⁺CD8⁺ T cells in the FD group than that in the control group, indicating the synergistic effect of chemotherapy and immunotherapy on the tumor.

Discussion

Fe₃O₄ is a type of ferromagnetic material, while the nano-spheres based on Fe₃O₄ have a wide range of applications in biomedical and bioengineering fields due to their special physical, chemical, and biological properties [30]. Therapeutic drugs based on Fe₃O₄ nanoparticles are gradually attracting increasing attention in medical areas due to its integration of both imaging and therapeutic functions into a single nanoagent, enabling accurate cancer diagnosis and personalized medical treatment as well as real-time monitoring of drug distribution and evaluation of treatment outcomes [31, 32]. In recent years, with the rapid development of nanotechnology, the diagnosis and treatment of multifunctional and multimodal nanocarriers in the field of biomedical applications have shown a significant potential. Several types of iron oxide nanoparticles have been evaluated in preclinical and clinical trials with some already available as commercial products on the market. It is reasonably expected that a variety of Fe₃O₄-based diagnosis-to-drug complexes will be routinely used in clinical practice in the coming decades [33]. In our study, we developed a PD-L1@Fe₃O₄-DOX (FPD) nano-drug delivery system. Our results provide a novel integrated multifunctional nanosystem for medical diagnosis and treatment.

With increasing incidence and survival of TNBC, the discovery of novel therapeutic approaches is required to advance the treatment outcomes of patients [34]. Recently, nanoparticles are regarded as a novel solution to improving the therapeutic efficacy and reducing the systemic toxicity in antitumor treatment owing to their smaller size, shorter circulation time, and larger modified surface area in comparison to the traditional materials. Different from traditional drug-delivery system, the nanoparticles with modified surface can bind to receptors expressed at the target site and can be incorporated into cancer cells through its pharmacological action [35]. In routine clinical practice, the combination chemotherapy for breast cancer is often the preferred treatment plan. The benefits of multidrug combination therapy include increasing or maintaining efficacy by reducing dose, reducing toxicity, and delaying drug resistance. Due to these advantages, combination therapy has now become a routine application strategy in clinical practice [36, 37]. In our study, we synthesized FPD through modification to achieve the effect of combination therapy of tumor in mice. We further performed the ICP-MS assay, the Prussian blue staining assay, the Hoechst 33342/PI fluorescence double staining assay, and the ROS assay to investigate the cellular uptake, targeting, and killing ability of FPD cells. The results showed that, on the one hand, Fe₃O₄ (before modification) and FP only attached to PD-L1 antibody showing no evident damage to cells with sound biosafety; on the other hand, FPD combined with PD-L1 antibody and chemotherapy drug DOX showed an increased killing effect on tumor cells, with FPD more efficient than FD.

We performed MRI imaging to evaluate the PD-L1 as a biomarker for targeted TNBC imaging and therapy. Our results revealed a relatively lower signal for FPD in the tumor regions compared to Fe_3O_4 , indicating that FPD can specifically target the 4T1 tumors and accumulate at the tumor site for a longer time than the non-targeted Fe_3O_4 control. For MRI, the Fe chelates are still commonly used as the agents for tumor diagnosis [38, 39]. In contrast, FPD significantly enhanced the efficacy of nanoparticles as MRI contrast agents.

Finally, we used Fe_3O_4 to load DOX to significantly improve the biosafety. On the one hand, the nanospheres may protect the DOX from the degradation by relevant immune cells or enzymes in the blood, though we note that more studies are needed to verify these speculations. On the other hand, nanospheres enhance targeting, effectively prevent DOX deposition in other organs, and improve biosafety, suggesting that the doses of DOX can be increased in the treatments.

In conclusion, the FPD nano-drug loading and monitoring system synthesized in this study can reduce the damage of DOX chemotherapy to normal tissues by targeting specifically the PD-L1 protein on the surface of tumor cells, showing the combined immunochemotherapy, simultaneously achieving the real-time MRI monitoring as well.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

JZ and ML performed the in vitro experiments and the in vivo experiments; JD and HG analyzed the data; DY designed the project. All authors reviewed the manuscript.

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Availability of data and materials

All data used and analyzed during this study are available from this published article and its supplementary information files. The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animal care protocols and experiments involved in this study were in accordance with protocols approved by the Ethics Committee of Qilu Hospital of Shandong University.

Consent for publication

All authors concur with the submission and publication of this paper.

Competing interests

The authors declare no competing interests.

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Figures

Figure 1

Characterization of superparamagnetic Fe₃O₄ based nanoparticles. (a) TEM image of Fe₃O₄ nanoparticles. (b) HRTEM image of Fe₃O₄ nanoparticles. (c) TEM image of PD-L1@Fe₃O₄-DOX (FPD) nanoparticles. (d) XRD pattern of Fe₃O₄ nanoparticles. (e) Saturation magnetization curve of Fe₃O₄ nanoparticles. (f) FTIR spectra of Fe₃O₄ and Fe₃O₄-DMSA nanoparticles. (g) UV-Vis absorbance spectrum. (h) Zeta potential value. (i) Size distributions of Fe₃O₄ nanoparticles aqueous after conjugation with DOX and PD-L1 antibody, respectively.

Figure 2

Targeting of nanoparticles in cells. (A) PD-L1 expression on 4T1 cells. PD-L1 was marked in green. (B) MRI imaging of cells after ingesting with different groups of nanoparticles. (C) Quantitation of MRI imaging of cells in (B). (D) Prussian blue staining of Fe_3O_4 and FPD cells at different concentrations. (E) ICP-MS quantitative images of Fe_3O_4 and FPD cells at different concentrations. (F) TEM images of cells before and after ingestion of different groups of nanoparticles. Symbol “***” indicates significant difference set at $P < 0.001$ based on Student’s t test.

Figure 3

Determination of Fe_3O_4 cytotoxicity and FPD efficacy in vitro. (A) The cell viabilities of MCF-10A and 4T1 cell lines incubated with Fe_3O_4 at different content (0, 25, 50, 100, and 200 $\mu\text{g/ml}$) for various time points (6, 12, 24, and 48 h). Fluorescence (B) and quantitative images (C) of apoptosis detected by Hoechst 33342/PI double staining. ROS was quantified (D) by DCFH-DA staining (E). Data are presented as mean $\pm$ SD. Symbols *, **, and *** indicate the significant differences based on Student's t test set at P

< 0.05, P < 0.01, and P < 0.001, respectively. Black asterisks indicate the comparison with Fe₃O₄-treatment group. Red asterisk indicates the comparison with the FPD-treatment group.

Figure 4

MRI of FPD both in vitro and in vivo. (A) T2-weighted MRI images and (B) relaxivity measurement at series concentrations of FPD in vitro. (C) Quantification of the MRI relative signal decreased of the tumor areas after the in vivo MRI. (D) T2-weighted MRI of the mice with 4T1 tumor before and 24 h after intravenous injection of FPD and Fe₃O₄ based on a 3.0 T small-animal MRI scanner. The red dashed

circles marked the tumor areas. Symbols *, **, and *** indicate the significant differences based on Student's t test set at $P < 0.05$, $P < 0.01$, and $P < 0.0001$, respectively. Data are presented the mean $\pm$ SD of at least five animals for each group.

Figure 5

Evaluation of the therapeutic effects with different treatments. (A) Schematic presentation of the animal experiment schedule. (B) tumor volume measurement at different time points for 15 days. Data are presented as the mean $\pm$ SE. (C) The Kaplan–Meier survival plots of the mouse survival rate after different treatments. (D) The mouse body weights of the mice with 4T1 tumor measured after different treatments for 15 days. Symbols *, **, and *** indicate the significant differences based on Student's t test set at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. Data represent the mean $\pm$ SD of at least five animals for each group.

Figure 6

FPD promotes tumor cell apoptosis and reduces organ toxicity. (A) The staining under microscope. Bar = 50 μ m. (B) Statistical analysis of data presented in (A). The number of TUNEL positive cells in the tumor of the group FPD was significantly higher than that of other groups ($P < 0.01$). (C) TUNEL staining images of heart, liver, spleen, and kidney. (D) Positive cell count based on the images in (C). (E) q-PCR was performed to detect the expression levels of genes caspase-3, Bax, and Bcl-2. Symbols *, **, and ***

indicate the significant differences based on Student's t test set at $P < 0.05$, $P < 0.01$, and $P < 0.0001$, respectively. Data are presented as the mean $\pm$ SD of at least five animals for each group.

Figure 7

Efficacy of immunotherapy in vivo. (A) Immunohistochemical staining of breast cancer tumor tissues for PD-L1 expression. (B) Histological analysis of breast cancer tumor sections with Prussian blue staining in the Fe₃O₄ group (left) and FPD group (right). (C) Flow cytometry analysis of CD3+CD8+ T cells under different treatments. (D) Quantitative maps of data presented in (C). Symbols * and *** indicate the significant differences based on Student's t test set at $P < 0.05$ and $P < 0.001$, respectively. Data are presented as the mean $\pm$ SD of at least five animals for each group.

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