EGFR-targeted delivery of DOX-loaded Fe$_3$O$_4$@polydopamine multifunctional nanocomposites for MRI and antitumor chemo-photothermal therapy

Abstract: Multifunctional nanocomposites that have multiple therapeutic functions together with real-time imaging capabilities have attracted intensive concerns in the diagnosis and treatment of cancer. This study developed epidermal growth factor receptor (EGFR) antibody-directed polydopamine-coated Fe$_3$O$_4$ nanoparticles (Fe$_3$O$_4$@PDA NPs) for magnetic resonance imaging and antitumor chemo-photothermal therapy. The synthesized Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs revealed high storage capacity for doxorubicin (DOX) and high photothermal conversion efficiency. The cell viability assay of Fe$_3$O$_4$@PDA-PEG-EGFR NPs indicated that Fe$_3$O$_4$@PDA-PEG-EGFR NPs had no cell cytotoxicity. However, Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs could significantly decrease cell viability (~5% of remaining cell viability) because of both photothermal ablation and near-infrared light-triggered DOX release. Meanwhile, the EGFR-targeted Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs significantly inhibited the growth of tumors, showing a prominent in vivo synergistic antitumor effect. This study demonstrated the potential of using Fe$_3$O$_4$@PDA NPs for combined cancer chemo-photothermal therapy with increased efficacy.

Keywords: Fe$_3$O$_4$ nanoparticles, polydopamine, chemo-photothermal therapy, multifunctional nanocomposites, DOX

Introduction

Recently, multifunctional nanocomposites that combine diagnostic and therapeutic functions have drawn an increasing concern. Ideally, in addition to safety and nontoxicity, these multifunctional nanocomposites should also have high drug-loading efficiencies and real-time imaging capabilities. More importantly, these nanocomposites should lead to lower toxicity to normal cells. Magnetic Fe$_3$O$_4$ nanoparticles (NPs) is one of the inorganic-based nanomaterials approved for clinical use, which showed great biocompatibility and has attracted significant attention due to their unique characteristics such as magnetic resonance imaging (MRI) response to an external magnetic field. Later, many biocompatible polymers, including chitosan, polyethylene glycol (PEG), and dextran, have been used to be coated in Fe$_3$O$_4$ NPs in order to further improve the properties of Fe$_3$O$_4$ NPs.

As a major pigment of naturally occurring melanin, polydopamine (PDA) is highly biocompatible and biodegradable. Due to its nature, PDA has been widely used, to be coated in NPs, for various biomedical applications. Meanwhile, by dispersing the as-prepared cores in an alkaline dopamine solution, PDA could spontaneously form a conformal layer through in situ polymerization. In addition, PDA has rich functional groups such as amino and catechol, which can facilitate the further functionalization
of PDA-based NPs with biomolecules and can improve the stability and functionality of NPs. Recent evidence has demonstrated that PDA-coated gold nanoshells could be stable within the cells of liver and spleen for at least 6 weeks.\(^\text{10}\)

Photothermal therapy (PTT) is a new noninvasive cancer treatment technique, which is mediated by inorganic NPs responsive to near-infrared (NIR) light and could convert light energy into thermal energy.\(^\text{11}\) As a new PTT agent, in addition to excellent biocompatibility in vitro and in vivo, PDA also had the strong NIR absorbance and high photothermal conversion efficiency (up to 40%).\(^\text{12,13}\)

This study fabricated doxorubicin (DOX)-coated \(\text{Fe}_3\text{O}_4@\text{PDA}\) NPs that could be simultaneously used for NIR-response PTT, chemotherapy, and MRI. Although there were reports about the synthesis and applications of \(\text{Fe}_3\text{O}_4@\text{PDA}\) NPs, to the best of our knowledge, the combination of PTT with chemotherapy (DOX) for the applications of antibody-targeted \(\text{Fe}_3\text{O}_4@\text{PDA}\) NPs has not been explored until now. Several reports showed that the tumors could not be completely eradicated by PTT alone due to the absorption and scattering of the NIR light by the biological tissues.\(^\text{16,17}\)

Then, integration of an efficient chemotherapy drug with PTT (termed chemo-photothermal therapy) is promising for enhanced and optimized antitumor efficacy.\(^\text{18–20}\) DOX, as an aromatic chemotherapy drug, can be effectively loaded onto the PDA shell via \(\pi-\pi\) stacking. Using DOX-loaded NPs as the model system, it was confirmed that the intracellular uptake of \(\text{Fe}_3\text{O}_4@\text{PDA-PEG-EGFR-DOX}\) NPs and the release of DOX from \(\text{Fe}_3\text{O}_4@\text{PDA-PEG-EGFR-DOX}\) NPs localized inside the cells could be stimulated by NIR laser irradiation due to mild photothermal heating. The combined chemo-photothermal therapy achieved excellent synergistic therapeutic efficacy both in vitro and in vivo. The \(\text{Fe}_3\text{O}_4@\text{PDA-PEG-EGFR}\) NPs could further be utilized as the \(T^*_2\) contrast agent in MRI to track tumor development. The results of the present study promised the use of \(\text{Fe}_3\text{O}_4@\text{PDA}\) core–shell NPs for combined antitumor chemo-photothermal therapy and MRI.

**Materials and methods**

**Materials**

Iron acetylacetonate (Fe(acac)_3), 1,2-hexadecanediol, benzyl ether, oleyamine (OLA), oleic acid (OA), sodium dodecyl sulfate (SDS), dopamine hydrochloride (DP), 1-ethyl-3-[3-dimethylaminopropyl]carboximide hydrochloride (EDC), and N-hydroxysuccinimide (NHS) were purchased from Millipore-Sigma (Darmstadt, Germany). Dulbecco’s Modified Eagle’s Medium (DMEM) with high glucose and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). \(\text{NH}_2\text{-PEG-COOH}\) (molecular weight \(=2,000\) Da) was purchased from Seebio Biological (Shanghai, China). DOX hydrochloride was purchased from Sangon Ltd. (Shanghai, China). Anti-EGFR antibody was obtained from Ruiying Biological (Suzhou, China). Other reagents (analytical grade) were purchased from Beijing Chemical Reagents Company (Beijing, China) unless otherwise stated.

**Cells and animals**

The DLD-1 human colon cancer cell line was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10% FBS in a humidified 5% CO\(_2\) atmosphere at 37°C.

Female BALB/c nude mice (5–6 weeks old) were purchased from Vital River Company (Beijing, China) and were maintained under specific pathogen-free conditions. The animals were treated according to the ethical guidelines of Jilin University after obtaining approval from the Animal Welfare and Research Ethics Committee of Jilin University. The animal experiments were carried out following the internationally accepted animal care guidelines (EEC Directive of 1986; 86/609/EEC).

**Preparation of \(\text{Fe}_3\text{O}_4@\text{PDA-PEG-EGFR}\) NPs**

\(\text{Fe}_3\text{O}_4@\text{PDA-PEG-EGFR}\) NPs were prepared as shown in Figure 1. First, \(\text{Fe}_3\text{O}_4\) NPs were synthesized as reported previously.\(^\text{21}\) Second, to synthesize \(\text{Fe}_3\text{O}_4@\text{PDA}\) NPs, Tris-buffer (12 mL) was added into the as-prepared \(\text{Fe}_3\text{O}_4\) NPs and adjusted to pH 8.5, followed by adding different volumes of 0.03 M dopamine solution. The reaction mixture was incubated at room temperature for 3 h with the solution color gradually turning to dark brown, indicating in situ polymerization of dopamine. Last, the \(\text{Fe}_3\text{O}_4@\text{PDA}\) NPs were obtained by centrifugation at 10,000 rpm for 10 min and washed with deionized water.

\(\text{Fe}_3\text{O}_4@\text{PDA-PEG}\) NPs were synthesized as reported previously.\(^\text{22}\) Briefly, for PEG modification, the as-prepared \(\text{Fe}_3\text{O}_4@\text{PDA}\) (10 mg) was reacted with \(\text{NH}_2\text{-PEG-COOH}\) (30 mg) in 50 mL Tris-buffer (0.01 M, pH 8.1) overnight under vigorous stirring. Then, PEGylated \(\text{Fe}_3\text{O}_4@\text{PDA}\) (termed \(\text{Fe}_3\text{O}_4@\text{PDA-PEG}\)) was purified by centrifugation at 10,000 rpm and redispersed in deionized water.

For EGFR antibody bioconjugation, EDC (5 mmol/L) and NHS (12.5 mmol/L) were dissolved in phosphate-buffered saline (PBS) containing \(\text{Fe}_3\text{O}_4@\text{PDA-PEG}\) NPs (pH 5.0). After 20 min, mouse anti-human EGFR monoclonal antibody
was added to this solution. Then, the pH of the reaction solution was adjusted to 7.5. The reaction lasted for 4 h at 4°C. The antibody-bioconjugated NPs (termed Fe₃O₄@PDA-PEG-EGFR NPs) were isolated by centrifugation and redispersed in deionized water.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)
The prepared Fe₃O₄@PDA-PEG-EGFR NPs were separated by a 10% SDS-PAGE according to the method of Laemmli.²³

Immunofluorescence
DLD-1 cells were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 in PBS for 30 min. Then, the cells were washed and blocked with 5% bovine serum albumin for 30 min. Cells were incubated with the primary EGFR antibodies (1:100; Ruiying Biological) overnight at 4°C. Then, the cells were washed and incubated with goat anti-mouse immunoglobulin G conjugated with fluorescein isothiocyanate for 1 h at room temperature. 4,6-Diamidino-2-phenylindole (DAPI; 1:10,000; Beyotime, Shanghai, China) was used for nucleus staining. Images were detected by using a fluorescence microscope (IX5-RFACA; Olympus Corporation, Tokyo, Japan).

Characterization of Fe₃O₄@PDA-PEG-EGFR NPs
The morphology of NPs was analyzed by using transmission electron microscope (TEM; Hitachi H-800 Hitachi, Tokyo, Japan). The size and its distribution of NPs were determined by dynamic light scattering (DLS) (Malvern Zetasizer Nano Instrument; Malvern Instruments, Malvern, UK). The zeta potentials of the as-prepared NPs were measured by using a Zetasizer Nano ZS (Malvern Instruments). The Fourier transform–infrared (FT-IR) spectroscopy of NPs was performed by using a Bruker Vertex 70 FT-IR spectrometer (Bruker, Karlsruhe, Germany) in the range from 400 to 4,000 cm⁻¹. A vibrating sample magnetometer was used for characterizing the magnetic properties of the NPs.

Loading DOX on Fe₃O₄@PDA-PEG-EGFR NPs
Fe₃O₄@PDA-PEG-EGFR NPs (1 mg) were suspended in 10× Tris-buffer (0.5 mL) and mixed with DOX solution (2 mL, 1 mg/mL). After 24 h of continuous stirring in the
dark, the obtained nanocomposites (named as Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs) were purified by centrifugation and washed with deionized water. The loading weight of DOX was calculated as follows: $W = W_{\text{original DOX}} - W_{\text{DOX in supernatant}}$.

The amount of DOX was determined by using the calibration curve of DOX at the wavelength of 480 nm.

**pH- and photothermal-sensitive DOX release**

Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs were suspended in PBS at different pH, sealed in a dialysis bag (molecular weight cutoff = 8,000 Da), and then were immersed into PBS solution at pH 5.0 or 7.4 at 37°C with moderate shaking for different periods of time. At desired time intervals, DOX in the release medium was collected, then the concentrations were determined by UV−vis spectrometry. The release medium was replaced with an equal volume of fresh PBS. The 808 nm NIR laser-triggered DOX release experiments were conducted following the same procedure as mentioned earlier. At predetermined time intervals, the samples were irradiated with an 808 nm NIR laser (0.6 W/cm$^2$) for 6 min. DOX in the dialysis buffer released from NPs was collected before and after 808 nm NIR laser irradiation.

**In vitro photothermal experiments**

A series of Fe$_3$O$_4$@PDA-PEG-EGFR NPs aqueous solutions with different concentrations (0, 15, 30, 60, 120, and 150 µg/mL) were irradiated with an 808 nm NIR laser (0.6 W/cm$^2$) for 6 min. The solution temperature was measured every 1 min by a thermometer with a thermocouple probe submerged in the solution. To detect the thermal stability of Fe$_3$O$_4$@PDA-PEG-EGFR NPs, the samples were irradiated for 6 min every time, followed by natural cooling of temperature to room temperature for five cycles, and the temperature was recorded every 1 min.

**In vitro MRI of phantom**

Fe$_3$O$_4$@PDA-PEG-EGFR NPs aqueous solutions with various concentrations (0, 15, 30, 60, 120, and 240 µg/mL) were prepared in 1.5 mL eppendorf tubes and swirled for 3 min before MRI. The T$_2$-weighted MRI was measured with a GE Sigma 3.0-T MR imaging system (General Electric, Milwaukee, WI, USA). The imaging parameters were listed as follows: TR, 1,390.0 ms; TE, 13.8 ms, field of view, 50×50 mm; and slice thickness, 2.5 mm.

**Cellular uptake and internalization of Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs**

To study the cellular uptake and the intracellular distribution of Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs, DLD-1 cells were incubated with Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs (equal to DOX 5 µg/mL) for 6, 12, 24, and 36 h at 37°C. At predetermined time points after incubation, the cells were fixed with a 4% paraformaldehyde, and the cellular nuclei were stained with DAPI (1:10,000; Beyotime). The results were characterized by using a fluorescence microscope. In a separate experiment, DLD-1 cells were irradiated with the 808 nm NIR laser (0.6 W/cm$^2$) for 6 min after incubation with Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs (equal to DOX 5 µg/mL) dispersions for 12 h. Then, the cells were treated in accordance with the aforementioned method and characterized by using a fluorescence microscope.

**Cell viability assay**

Cell Counting Kit-8 (CCK-8) assays were carried out to evaluate the potential cytotoxicity of Fe$_3$O$_4$@PDA-PEG-EGFR NPs. First, DLD-1 cells were seeded in a 96-well cell-culture plate at 200 µL (1×10$^5$ cells/mL) per well and incubated for 24 h. Then, the medium was removed, and the suspensions of Fe$_3$O$_4$@PDA-PEG-EGFR NPs or Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs at selected concentrations (0, 6.25, 12.5, 25, 50, and 100 µg/mL) were added to the wells. The Fe$_3$O$_4$@PDA-PEG-EGFR NPs cells and Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs-stained cells were irradiated by an 808 nm NIR laser (0.6 W/cm$^2$) for 6 min and were incubated at 37°C for another 24 h. Later, 10 µL of CCK-8 was added to each well. After incubation for 2 h, the absorbance of each well was read on a Microplate Reader (ELx-800; BioTek Instruments, Winooski, VT, USA) at 450 nm. The relative cell viability (%) related to the control wells containing cell-culture medium was calculated by using the following equation: Cell viability (%) = $A_{\text{Test sample}} / A_{\text{Control}} \times 100\%$, where $A_{\text{Test sample}}$ and $A_{\text{Control}}$ were the absorbance of the test sample and the control, respectively.

**In vivo MRI**

To develop the tumor model, DLD-1 cells (100 µL, 1×10$^7$/mL) suspended in PBS were inoculated subcutaneously into the back of each mouse. For in vivo MRI, desired amounts of Fe$_3$O$_4$@PDA-PEG-EGFR NPs in PBS solutions (100 µL) were injected intravenously into the mouse through the tail vein. In vivo MRI was performed at predetermined time points after injection. The T$_2$-weighted MRI parameters were shown as follows: TR, 3,980.0 ms; TE, 99.0 ms; field of view, 400.0 mm; and slice thickness, 6.0 mm.

**In vivo chemo-photothermal therapy**

For chemo-photothermal therapy, when the tumors grew to 5–6 mm in diameter, the mice were randomly divided into
eight groups (n=5 per group), namely PBS treated, PBS with NIR laser treated, Fe₃O₄@PDA-PEG NPs treated, Fe₃O₄@PDA-PEG NPs with NIR laser treated, Fe₃O₄@PDA-PEG-EGFR NPs treated, Fe₃O₄@PDA-PEG-EGFR NPs with NIR laser treated, Fe₃O₄@PDA-PEG-EGFR-DOX NPs treated, and Fe₃O₄@PDA-PEG-EGFR-DOX NPs with NIR laser treated. Mice of each group were intravenously injected with 100 µL of PBS, Fe₃O₄@PDA-PEG NPs, Fe₃O₄@PDA-PEG-EGFR NPs, or Fe₃O₄@PDA-PEG-EGFR-DOX NPs. The mice were anesthetized with chloral hydrate (5%), and the tumors were irradiated with or without an 808 nm laser (0.6 W/cm²) for 6 min after 24 h injection. After treatments, the length and width of the tumors were tracked with a caliper every 2 days for 14 days. The tumor volume was calculated as follows: Tumor volume = (tumor length) × (tumor width)²/2. The relative tumor growth ratio was calculated as V/V₀, where V and V₀ were the tumor volumes on day 14 and day 0, respectively.

Histological analysis
BALB/c mice from the treatment group were killed, and the tumor tissues and other major organs including heart, liver, spleen, lung, and kidney were collected for analysis after various treatments. The frozen tissue slides were further stained with hematoxylin and eosin following the standard protocol and examined using a fluorescence microscope.

Statistical analysis
All values are expressed as the mean ± SD and analyzed by using Student’s t-test. P-values <0.05 were considered statistically significant.

Results and discussion
Preparation and characterization of Fe₃O₄@PDA-PEG-EGFR-DOX NPs
The Fe₃O₄@PDA-PEG-EGFR-DOX NPs were prepared as shown in Figure 1. Fe₃O₄@PDA NPs were synthesized through spontaneous in situ polymerization of PDA. After coating, the average size of Fe₃O₄@PDA NPs was determined by TEM and DLS. The average size of Fe₃O₄@PDA observed from TEM was ~60 nm (Figure 2A), whereas the average hydrodynamic diameter measured by DLS was ~91.2±31.1 nm (Figure 2B). Absorption bands, 3,421 cm⁻¹ (N-H stretching) and 1,524 cm⁻¹ (N-H bending) in the FT-IR spectrum of Fe₃O₄@PDA (as shown in Figure 2C) further confirmed the presence of PDA on Fe₃O₄. Then, the Fe₃O₄@PDA NPs thus obtained were sequentially modified with NH₂-PEG-COOH and EGFR antibody via covalent conjugation to form Fe₃O₄@PDA-PEG-EGFR NPs. The NH₂-PEG-COOH was conjugated onto the surface of Fe₃O₄@PDA NPs through the reaction between terminal amine group of NH₂-PEG-COOH and catechol group of PDA by a Schiff base reaction pathway. As illustrated in the FT-IR absorption spectrum (Figure 2C), the characteristic peaks of NH₂-PEG-COOH at 3,393 cm⁻¹ (N-H stretching), 1,635 cm⁻¹ (C-O stretching), and 1,091 cm⁻¹ (C-O-C stretching) indicated that NH₂-PEG-COOH had been successfully conjugated onto the Fe₃O₄@PDA NPs. The EGFR antibody was successfully conjugated to Fe₃O₄@PDA-PEG NPs, which was proved by SDS-PAGE analysis. As shown in Figure 2D, the Coomassie blue-stained protein band represented the free EGFR antibody and Fe₃O₄@PDA-PEG-NPs that were diffused in gel. For Fe₃O₄@PDA-PEG-EGFR NPs, the Coomassie blue-stained EGFR antibody colocalized with Fe₃O₄@PDA-PEG NPs, indicating that EGFR antibody was chemically linked to the surface of the Fe₃O₄@PDA-PEG NPs. In contrast, Fe₃O₄@PDA-PEG NPs have no corresponding band by Coomassie blue-stained gel analysis. All of these results suggested that Fe₃O₄@PDA-PEG-EGFR NPs had been successfully constructed.

Because of the presence of magnetic iron oxide core, the Fe₃O₄@PDA-PEG-EGFR NPs displayed strong magnetic property. When placed beside a magnet, the Fe₃O₄@PDA-PEG-EGFR NPs in aqueous solution was attracted by a magnet, which was almost unchanged without a magnet (Figure 3A). The strong superparamagnetism of Fe₃O₄@PDA-PEG-EGFR NPs was further revealed by the field-dependent magnetization hysteresis loop (Figure 3B). Fe₃O₄ NPs have been widely used as a T₂-contrast agent for MRI. As shown in Figure 3C and D, the T₂-weighted MR images exhibited increasingly darkening effect with the increase of Fe₃O₄@PDA-PEG-EGFR NPs concentration. This result suggested that Fe₃O₄@PDA-PEG-EGFR NPs could be used as a T₂-weighted MRI contrast agent.

Photothermal properties of Fe₃O₄@PDA-PEG-EGFR NPs
The photothermal conversion capability of Fe₃O₄@PDA-PEG-EGFR NPs was evaluated by irradiating Fe₃O₄@PDA-PEG-EGFR aqueous solution with an 808 nm NIR laser at 0.6 W/cm². As shown in Figure 4A, the temperature strikingly rose with increasing concentration of Fe₃O₄@PDA-PEG-EGFR NPs, following a time- and concentration-dependent manner. In particular, at an Fe₃O₄@PDA-PEG-EGFR NPs concentration of 150 µg/mL, the solution temperature could reach 50°C within 6 min irradiation, which is the temperature required to kill cancer cells. The thermal stability of Fe₃O₄@PDA-PEG-EGFR NPs was assessed by NIR laser irradiations at 0.6 W/cm² for
Figure 2 Confirmation of the bioconjugation of EGFR antibody to Fe₃O₄@PDA NPs.

Notes: (A) TEM image and (B) size distribution histogram of the Fe₃O₄@PDA NPs. (C) FT-IR spectra of Fe₃O₄@PDA and Fe₃O₄@PDA-PEG. (D) SDS-PAGE analysis of Fe₃O₄@PDA-PEG-EGFR and EGFR antibody. The Coomassie blue-stained gel analysis revealed the successful cross-linking of EGFR antibody molecules on the surface of the Fe₃O₄@PDA-PEG NPs. Lane 1, EGFR antibody; lanes 2 and 3, the bioconjugated Fe₃O₄@PDA-PEG-EGFR NPs; lane 4, protein molecular weight marker; lane 5, Fe₃O₄@PDA-PEG NPs.

Abbreviations: FT-IR, Fourier transform–infrared; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TEM, transmission electron microscope; PDA, polydopamine; PEG, polyethylene glycol; NP, nanoparticle.

6 min, followed by natural cooling of temperature to room temperature for five cycles. After five cycles of laser irradiation, there was no noticeable attenuation in the thermal conversion efficiency of the Fe₃O₄@PDA-PEG-EGFR NPs (Figure 4B). The remarkable photothermal conversion efficiency and thermal stability indicated that the Fe₃O₄@PDA-PEG-EGFR NPs could be used as an excellent candidate for PTT applications.
Drug loading and releasing on $\text{Fe}_3\text{O}_4@\text{PDA-PEG-EGFR-DOX}$ NPs

Chemotherapy drug DOX is mixed with $\text{Fe}_3\text{O}_4@\text{PDA-PEG-EGFR}$ NPs in Tris-buffer (pH 8.0) overnight. As shown in Figure 5A, the UV-vis absorption spectrum of $\text{Fe}_3\text{O}_4@\text{PDA-PEG-EGFR-DOX}$ NPs showed that the DOX characteristic peak shifted from 480 to $\sim$500 nm, confirming the loading of DOX onto $\text{Fe}_3\text{O}_4@\text{PDA-PEG-EGFR}$ NPs. The maximal DOX loading ratio (DOX:$\text{Fe}_3\text{O}_4@\text{PDA-PEG-EGFR}$ NPs, w/w) was estimated to be $\sim$80% (data not shown). Then, the drug-releasing capability of $\text{Fe}_3\text{O}_4@\text{PDA-PEG-EGFR-DOX}$ NPs was examined at pH 5.0 and...
7.4. As shown in Figure 5B, within 36 h, ∼25% of DOX was released from the Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs at pH 5.0 compared with 6% of DOX release at pH 7.4. The amount of DOX released from Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs at pH 5.0 was approximately fourfold higher than that at pH 7.4. The protonation of the amino group in the DOX molecule gave DOX a positive charge and thus enhanced the hydrophilicity to trigger drug release at a lower pH.

To investigate the photothermal influence on DOX release, Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs suspended in PBS at pH 5.0 or 7.4 were irradiated under an 808 nm NIR laser (0.6 W/cm$^2$, 6 min). As shown in Figure 5C, a significant thermo-triggered burst release of DOX from Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs was observed at pH 5.0. In comparison, only limited DOX was released at pH 7.4 under the same condition. The pH-dependent NIR-triggered drug release processes enabled regulation of intracellular drug release and minimizing of the side effects of chemotherapy drugs.

Cellular uptake of Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs

The cellular uptake and internalization of Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs for the intracellular delivery of DOX were investigated by using DLD-1 human colon cancer cells. First, the level of EGFR expression on the surface of DLD-1 cells was detected by immunofluorescence (Figure 6A). The DLD-1 cells were incubated with Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs for 6, 12, 24, and 36 h. With incubation time increasing from 6 to 36 h, the red fluorescence of DOX was gradually distributed in the cytoplasm and in the nuclei of the Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NP-treated cells (Figure 6B). This result indicated that DOX molecules were released from Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs taken up by DLD-1 cells. In addition, the DOX released from Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs showed slow nuclear clustering because DOX was first released in the...
Figure 6 DOX release of Fe₃O₄@PDA-PEG-EGFR-DOX NPs under remote optical controls.

Notes: (A) The level of EGFR expression on the surface of DLD-1 cells was examined by immunofluorescence using an anti-EGFR antibody (green). Scale bar is 50 µm. (B) Fluorescence images of DLD-1 cells treated with Fe₃O₄@PDA-PEG-EGFR-DOX NPs for various times indicated. Images were taken from bright-field mode, DOX channel (red), and DAPI channel (blue), respectively. Scale bar is 50 µm. (C) Fluorescence images of DLD-1 cells incubated with Fe₃O₄@PDA-PEG-EGFR-DOX NPs with or without an 808 nm laser irradiation at 0.6 W/cm² for 6 min. Scale bar is 50 µm.

Abbreviations: DOX, doxorubicin; DAPI, 4,6-diamidino-2-phenylindole; PDA, polydopamine; PEG, polyethylene glycol; NP, nanoparticle.
cytotoxicity of Fe₃O₄@PDA-PEG-EGFR-DOX NPs. In contrast, the cell viabilities were sharply decreased when DLD-1 cells were incubated with Fe₃O₄@PDA-PEG-EGFR NPs in the presence of an 808 nm NIR laser irradiation (0.6 W/cm²) for 6 min (only 30% of remaining cell viability). Moreover, compared with Fe₃O₄@PDA-PEG-EGFR NPs, the inhibition effect of Fe₃O₄@PDA-PEG-EGFR-DOX NPs at an equivalent concentration appeared much stronger whether or not with NIR laser irradiation. These results suggested that Fe₃O₄@PDA-PEG-EGFR-DOX NPs had a prominent chemo-photothermal antitumor effect.

**In vivo targeting MRI of tumors**

The excellent in vitro MRI contrast performance of Fe₃O₄@PDA-PEG-EGFR NPs inspired us to study their applicability in vivo. Mice bearing DLD-1 tumors were intravenously injected with Fe₃O₄@PDA-PEG-EGFR NPs (200 µL, 100 µg/mL) and imaged by a 3.0-T clinical MR scanner at 0, 12, and 24 h. A strong darkening effect in the tumor area was observed in T₂-weighted MR images at 24 h after injection (Figure 8), suggesting that Fe₃O₄@PDA-PEG-EGFR NPs could be used for MRI-guided cancer therapy.

**Chemo-photothermal therapy**

Because of the prominent antitumor effect in vitro, the inhibiting tumor effect of Fe₃O₄@PDA-PEG-EGFR-DOX NPs was evaluated in vivo. The mice were divided into eight randomized groups and were first treated through intravenous injection of different materials. After treatment, the tumor sizes in different groups of mice were measured every 2 days for a period of 14 days. As shown in Figure 9A and B, tumors in control groups, including PBS injection with or without laser irradiation, as well as Fe₃O₄@PDA-PEG injection without laser irradiation, all showed obvious growth. The growth of tumors was slightly increased by Fe₃O₄@PDA-PEG-EGFR-DOX NPs without laser irradiation, which is consistent with the previous reports that DOX at such a low dose could not effectively suppress tumor growth. Importantly, more obvious tumor inhibiting effect was achieved from the group with Fe₃O₄@PDA-PEG-EGFR-DOX NPs injection and NIR laser irradiation during the 14 days. These in vivo results strongly demonstrated that Fe₃O₄@PDA-PEG-EGFR-DOX NPs were more efficient than monotherapy alone for ablation of tumors.

The potential toxicity of the NPs had been a major drawback for practical applications in vivo. In this experiment, it was found that mice in all test groups, especially for groups treated with Fe₃O₄@PDA-PEG-EGFR-DOX...
Figure 8 In vivo $T_2$-weighted MR images.

Notes: $T_2$-weighted MR images of mice after intravenous injection with $\text{Fe}_3\text{O}_4@\text{PDA-PEG-EGFR}$ NPs at 0, 12, and 24 h. Red circle indicates tumor position.

Abbreviations: MR, magnetic resonance; PDA, polydopamine; PEG, polyethylene glycol; NP, nanoparticle.

Figure 9 In vivo evaluation of chemo-photothermal therapeutic effect.

Notes: (A) Tumor growth curves of different groups of mice after treatment (day 14). The tumor volumes were normalized to their initial sizes. (B) Photographs of the tumors collected from different groups of mice after 14 days of treatment with (a) $\text{Fe}_3\text{O}_4@\text{PDA-PEG-EGFR-DOX} + \text{laser}$, (b) $\text{Fe}_3\text{O}_4@\text{PDA-PEG-EGFR} + \text{laser}$, (c) $\text{Fe}_3\text{O}_4@\text{PDA-PEG-EGFR} + \text{laser}$, (d) $\text{PBS} + \text{laser}$, (e) $\text{Fe}_3\text{O}_4@\text{PDA-PEG-EGFR-DOX}$, (f) $\text{Fe}_3\text{O}_4@\text{PDA-PEG-EGFR}$, (g) $\text{Fe}_3\text{O}_4@\text{PDA-PEG}$, and (h) $\text{PBS}$. (C) Histology analysis of the major organs of mice after 30 days of treatment, from the different groups: (a) PBS group and (b) $\text{Fe}_3\text{O}_4@\text{PDA-PEG-EGFR-DOX}$ irradiation with an 808 nm laser at 0.6 W/cm$^2$.

Abbreviations: PBS, phosphate-buffered saline; PDA, polydopamine; PEG, polyethylene glycol; NP, nanoparticle; DOX, doxorubicin.
NPs and NIR laser irradiation, behaved normally without a significant decrease in body weight (data not shown). The maintained body weight demonstrated that there was no noticeable toxicity of the prepared NPs in vivo. In addition, histological analysis of the major organs showed that there was no obvious tissue damage after 30 days of chemo-photothermal therapy, further confirming the low toxicity of the NPs in vivo (Figure 9C).

Conclusion
In summary, the multifunctional Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs capable of simultaneous chemo-photothermal therapy and MRI have been successfully designed and synthesized. DOX could be physically adsorbed on Fe$_3$O$_4$@PDA-PEG-EGFR NPs with high drug-loading ratios via π–π stacking. The resultant NPs exhibited pH and heat-responsive DOX release. When the NPs were uptaken into the DLD-1 cells, the release of the DOX could be prompted by the NIR irradiation. Meanwhile, the cells were killed by the released DOX and the local heating under the NIR irradiation. The tumor growth was effectively inhibited by enhanced DOX release of the Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs under the NIR irradiation and the NIR-induced PTT. The results of this study demonstrated that Fe$_3$O$_4$@PDA-PEG-EGFR-DOX NPs possessed sensitive drug release and prominent chemo-photothermal synergistic therapy effects for tumor inhibition and eradication.

Precision medicine is altering the traditional treatment methods for patients with cancer. Therefore, EGFR antibody-targeted Fe$_3$O$_4$@PDA-PEG-EGFR-DOX theranostic NPs are a promising receptor-targeted drug delivery system for the individual treatment of EGFR-overexpressed colon cancer.

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Disclosure
The authors report no conflicts of interest in this work.

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