Construction of Multifunctional Fe₃O₄-MTX@HBc Nanoparticles for MR Imaging and Photothermal Therapy/Chemotherapy

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Abstract

To accomplish effective cancer imaging and integrated therapy, the multifunctional nanotheranostic Fe₃O₄-MTX@HBc core-shell nanoparticles (NPs) were designed. A straightforward method was demonstrated for efficient encapsulation of magnetic NPs into the engineered virus-like particles (VLPs) through the affinity of histidine tags for the methotrexate (MTX)-Ni²⁺ chelate. HBc¹⁴₄-His VLPs shell could protect Fe₃O₄-MTX NPs from the recognition by the reticuloendothelial system as well as could increase their cellular uptake efficiency. Through our well-designed tactic, the photothermal efficiency of Fe₃O₄ NPs were obviously improved in vitro and in vivo upon near-infrared (NIR) laser irradiation. Moreover, Magnetic resonance imaging (MRI) results showed that the Fe₃O₄-MTX@HBc core-shell NPs were reliable T₂-type MRI contrast agents for tumor imaging. Hence the Fe₃O₄-MTX@HBc core-shell NPs may act as a promising theranostic platform for multimodal cancer treatment.

Key words: Virus-Like Nanoparticles; MRI; Chemotherapy; Photothermal Therapy; Theranostic

Introduction

Chemotherapy is one of the major categories of the medical discipline specifically devoted to pharmacotherapy for cancer. However, the current chemotherapeutic agents have many adverse effects on healthy tissues or normal cells.¹ Continuing improvement in the tumor specific multifunctional nanoparticles for targeting and efficient delivery of drugs to tumor cells is an area of intense research with the potential to revolutionize the treatment of cancer.²,³ Recently, the combination of photothermal and chemotherapy is widely applied in killing cancer cells by using nanoparticles, such as nanosized graphene oxide,⁴ gold nanoparticles,⁵ plasmonic copper sulfide nanocrystals,⁶ folate-lipid-poly(lactic-co-glycolic acid) lipid-polymer nanoparticles⁷ and so on.

As a major class of nanoparticles, magnetic nanoparticles have been examined extensively for applications in cancer therapy due to their ultra-small size, biocompatibility and magnetic properties.⁵,⁹ More recently, clustered Fe₃O₄ magnetic nanoparticles have been reported to possess broad optical
absorption in the near-infrared (NIR) range and have further been used as a class of photosensitizers for tumor photothermal therapy (PTT).\textsuperscript{10} Compared with other photosensitive nanomaterials, Fe\textsubscript{3}O\textsubscript{4} nanoparticles possess various advantages, such as: 1) superior biocompatibility; 2) biodegradability (Fe\textsubscript{3}O\textsubscript{4} nanoparticles can be degraded into iron ions \textit{in vivo} and excess iron ions can be transferred into ferritin proteins for iron storage and detoxification), and 3) good superparamagnetic properties make them as excellent contrast agents for magnetic resonance imaging (MRI).\textsuperscript{11,12} However, due to the easy agglomeration and the limited functional groups of Fe\textsubscript{3}O\textsubscript{4} nanoparticles, a suitable material coating, such as silica, polyethylene glycol (PEG), bovine serum albumin (BSA) and so on, is always necessary to improve the situations and is of significance for bioapplications.\textsuperscript{13-15}

Lately, virus-like particles have attracted much attentions because of their potential to be a new drug delivery system for clinical therapy.\textsuperscript{16} The VLPs are multimeric protein assemblies that can serve as robust synthetic carriers due to the ability to encase nucleic acids or other small molecules by self-assembling in proper conditions.\textsuperscript{17} The protein shells of VLPs are steady to protect the cargos from quick releasing in blood circulation and can be chemically and genetically readily modified by genetic engineering or chemical methods.\textsuperscript{18} The size of VLPs are commonly between 18 - 100 nm in diameter, makes them easily internalized by cells and biodegradable with extremely low cytotoxicity. The VLPs exhibit the potential to be a safe drug delivering system.\textsuperscript{19} VLP-based core-shell nanoparticles have been widely applied in biomedical applications. For example, Zhang Y. et al. have prepared quantum dots (QDs) loaded HIV-1 based lentivirus for tracking virus infection.\textsuperscript{20} Fang PY. et al. have encapsulated functional RNAs into bacteriophage Q\textbeta{} VLPs to attenuate cell proliferation and promote mortality of brain tumor cells.\textsuperscript{21} Moreover, Li C. et al. have constructed Ag\textsubscript{2}S QDs loaded simian virus 40 VLPs for real-time \textit{in vivo} NIR-II imaging.\textsuperscript{22} However, the integrated single function of the developed VLPs based core-shell nanoparticles limited their efficacy for tumor imaging and therapy. Therefore, it is vital to develop multifunctional VLPs based nanoprobe for tumor imaging and therapy.

The hepatitis B virus core antigen (HBc) could spontaneously assemble into an icosahedral particle in all viable prokaryotic and eukaryotic recombinant expression systems.\textsuperscript{23} Herein, we designed and developed a novel magnetic drug delivery system (Fe\textsubscript{3}O\textsubscript{4}-MTX@HBc), in which MTX was chemically conjugated to Fe\textsubscript{3}O\textsubscript{4} nanoparticles and subsequently embedded into a shell of hepatitis B virus core protein (HBc) VLPs. As a natural nanocarrier, HBc presented empty interior space that can enhance the stability and biocompatibility of the nanodrugs. Moreover, the HBc VLPs can be easily modified with functional groups using the methods of genetic or chemical engineering.\textsuperscript{24-26} Owing to these merits, HBc VLPs can be a promising candidate as nanocarriers.

The detailed synthetic procedures and potential applications for this drug delivery system were illustrated in Scheme 1. The multifunctional Fe\textsubscript{3}O\textsubscript{4}-MTX NPs still hold the photothermal and magnetic properties after modified MTX on the surface. The viral nature and the essence of protein of HBc\textsubscript{144}-His VLPs could facilitate the internalization of Fe\textsubscript{3}O\textsubscript{4}-MTX by the cancer cells. The presence of VLPs shell could also provide a protective layer for drug molecules and magnetite nanoparticles from the recognition by the reticuloendothelial system, therefore allowing drugs to be administered over prolonged periods.

**Materials and Methods**

**Materials**

Ferric chloride (FeCl\textsubscript{3}), anhydrous sodium acetate (NaOAc), and hexahydrate nickel chloride (NiCl\textsubscript{2}-6H\textsubscript{2}O) were purchased from Shantou Xilong Chemical Factory (Guangdong, China). Dimeric-octosuccinic acid (DMSA), phosphotungstic acid (TPA), dicyclohexyl carbodiimide (DCC) and MTX were purchased from Sigma-Aldrich (ST. Louis, USA), N-hydroxysuccinimide (NHS) were purchased from Solarbio (Beijing, China). Diethylene glycol (DEG), glycerol, and dimethyl sulfoxide (DMSO) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Urea, glycine, tris(hydroxymethyl) aminomethane (Tris), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and Annexin V-FITC/PI Apoptosis Detection Kit were purchased from KeyGen Biotech Co. Ltd. (Nanjing, China). RPMI 1640 culture medium and penicillin-streptomycin were purchased from Biological Industries (Beit Ahemeq, Israel). Fetal bovine serum (FBS) and trypsin were purchased from Invitrogen (California, USA). All chemicals were used without further purification. Ultrapure water (18.2 M\textsubscript{2} cm) were obtained from Milli-Q Water Purification System.

**Preparation and characterization of Fe\textsubscript{3}O\textsubscript{4} and Fe\textsubscript{3}O\textsubscript{4}-MTX@HBc**

**Synthesis of Fe\textsubscript{3}O\textsubscript{4}-MTX-Ni\textsuperscript{2+} nanocomposites**

Fe\textsubscript{3}O\textsubscript{4} NPs were prepared according to a former literature method.\textsuperscript{27} DMSA modified Fe\textsubscript{3}O\textsubscript{4} NPs
(11.7±1.6 nm in diameter) were obtained by the procedure as previously described.28 240 mg of the as-prepared Fe₃O₄-DMSA NPs were firstly re-dispersed in 20 ml of DMSO. 400 mg of DCC and 400 mg of NHS were then added and stirred for 30 min before magnetic separation. 24 mg of MTX and 24 ml of DMSO were added to the precipitation and stirred for 12 h before magnetic separation. The addition of DCC and NHS to the Fe₃O₄-DMSA could form a highly reactive intermediate (NHS-carboxylate), which could subsequently react with the free amino group on MTX.29 The supernatant was collected to determine unloaded MTX and subsequently to calculated the drug loading efficiency (% w/w). Afterwards, Fe₃O₄-MTX-Ni²⁺ complexes were acquired by mixing 23 mg of Fe₃O₄-MTX NPs with 240 mg of NiCl₂ (0.1 M aqueous solution) for 30 min and finally extracted by magnetic separation.

**Preparation of Fe₃O₄-MTX@HBc NPs**

HBc₁₄₄-His VLPs were prepared following the method from our former research with minor modifications.28 In brief, HBc₁₄₄-His VLPs were produced by *Escherichia coli* expression system and purified by ion-exchange column chromatography and molecular sieve chromatography. Herein, 23 mg of HBc₁₄₄-His VLPs were disassembled after incubating with 15 ml of the denaturant stock solution (2.5 M urea, 150 mM NaCl and 50 mM Tris-HCl) at 4 °C for 30 min. 23 mg of Fe₃O₄-MTX-Ni²⁺ was then added into the denaturant solution containing HBc₁₄₄-His subunits and vortexed at 4 °C for 10 min. The reassembly of VLPs was initiated at 4 °C by dialyzing the dissociated HBc₁₄₄-His subunits in an assembling buffer A (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% v/v glycerol, 1% w/v glycine). After 12 h, the dialysate was replaced by assembling buffer B (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% w/v glycine) and dialyzing for another 12 h. The Fe₃O₄-MTX-Ni²⁺-containing VLPs (Fe₃O₄-MTX@HBc) were finally acquired by magnetic separation and lyophilization (-20 °C, 12 h).

**Characterization**

The hydrodynamic radius of Fe₃O₄-MTX@HBc was determined by dynamic light scattering (DLS) measurements using Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, U.K.). Morphology and structure of the prepared NPs were characterized by a transmission electron microscopy (TEM, Tecnai G2 Spirit, FEI, USA) after the samples were negatively stained using 2 % TPA. Powder X-ray diffraction (XRD) patterns were collected using a German Bruker-axs D8-A25 X-ray diffractometer. The fourier transform infrared spectra (FT-IR) were recorded on a Fourier transform infrared spectroscope (Nicolet IS10, Thermo Fisher Scientific, USA). The drug loading efficiency of MTX in Fe₃O₄-MTX NPs was determined by high performance liquid chromatography (HPLC, separations module 2695, photo diode array detector 2996, Waters, USA). Photothermal experiments were conducted using an infrared reflector lamp (Hi-Tech optoelectronics, China) with a power density of 1.5 W·cm⁻². The temperature and thermal images were collected by infrared thermal camera (FLIR A35, FLIR, Sweden). A vibration sample magnetometer (Lake Shore 7404, Lake shore, USA) was used to measure the magnetic properties of the as-prepared samples. The concentration of Fe³⁺ was determined by inductively coupled plasma mass spectrometry (ICP-MS, 7500 CE, Agilent, USA).

**T₂-weighted MRI of Fe₃O₄-MTX@HBc**

T₂-weighted MRI was measured by a 7.0 T Varian MRI system (Agilent Technologies, USA) with a horizontal-bore Magnex.
magnet, equipped with 10 cm bore imaging gradients (40 G cm⁻¹). Different concentrations of Fe in Fe₃O₄-MTX@HBc (0.18, 0.37, 0.74, 1.48, and 2.97 mg·mL⁻¹) were suspended in 0.1% agarose for MRI. The testing parameters were adopted: TR (repetition time) = 2000 ms, TE (echo time) = 60 ms, matrix size = 192 × 192, slice thickness = 2 mm and FOV = 80 × 50. By fitting the reciprocal of T₂ relaxation time (s⁻¹) against the concentration of Fe in a line, the r₂ relativity values were derived from the slope of the formula representing the line.

For in vitro T₂-weighted MRI, 4T1 cells were incubated with culture media containing Fe₃O₄-MTX@HBc (200 μg·mL⁻¹) and equal amount of Fe₃O₄-MTX NPs for 1 h, 2 h and 3 h, respectively. The cells were harvested and washed with PBS to remove the free NPs. Then 10⁷ cells were suspended in 0.1% agarose for in vitro MRI. The testing parameters were adopted: TR = 2500 ms, TE = 11 ms, matrix size = 256 × 256, slice thickness = 1 mm, and FOV = 40 × 40.

In the T₂-weighted MRI of tumors in vivo, the murine breast cancer 4T1 subcutaneous xenograft tumor bearing mice (tumor size: ~ 400 mm³) were used. The MRI were performed before and after 30 min post of the intratumoral injection of Fe₃O₄-MTX@HBc (100 μL, 4 mg·mL⁻¹). The transverse plane MR images were acquired using the following parameters: TR = 2000 ms, TE = 60 ms, matrix size = 128 × 128, slice thickness = 2 mm, and FOV = 40 × 40.

In Vitro Photothermal effect of Fe₃O₄-MTX@HBc NPs

The murine breast cancer 4T1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units·mL⁻¹ penicillin and 0.1 mg·mL⁻¹ streptomycin, at 37 °C in 5% CO₂. After 4 h of incubation with Fe₃O₄-MTX@HBc NPs (200 μg·mL⁻¹) or normal medium (control), the cells were collected in vials. The cell pellets were irradiated for 5 min with an 808 nm laser at a power of 2 W·cm⁻² (facula 0.5 cm). The temperature and thermal images were collected with an infrared thermal camera (FLIR A35, FLIR, Sweden). After the cells were seeded in 96-well plates with 5000 cells/well and cultured for 12 h, Fe₃O₄-MTX@HBc NPs with different concentrations (25, 50, 100, 200, and 300 μg·mL⁻¹) were added to the wells, respectively. For photothermal treatments, the cells were treated with 5 min of irradiation of 808 nm laser light at power density (2 W cm⁻², facula 1.5 cm). After the cells were incubated at 37 °C for 24 h, 20 μL of MTT (5 mg·mL⁻¹ in PBS) was added into each well and incubated for another 4 h. The medium in each well was replaced by 200 μL of DMSO after the 4 h of incubation, and the plates were gently shaken for 0.5 h to dissolve the formazan precipitate. The cells were followed by determining absorbance at 490 nm using a spectrophotometric microplate reader (Infinite 200 pro, Tecan, Switzerland).

4T1 cells were seeded in 12 well plates at 1×10⁵ cells per well. After 12 h of incubation, 200 μg·mL⁻¹ of Fe₃O₄-MTX@HBc NPs was added to cells for another 6 h of incubation before exposed to NIR laser (808 nm, 2 W·cm⁻²) for 5 min. Cells incubated with normal medium were used as control group. Annexin V-FITC/PI Apoptosis Detection Kit was used to analyze cell apoptosis by flow cytometry (BD Accun, Becton Dickinson Medical Devices, USA) after 12 h of incubation followed by the standard protocols.

In Vivo Biodistribution analysis of Fe₃O₄-MTX@HBc NPs

For in vivo biodistribution analysis, 100 μL of Fe₃O₄-MTX@HBc and Fe₃O₄-MTX NPs (both amount of 12 mg·kg⁻¹) were separately intratumorally injected into the murine breast cancer 4T1 subcutaneous xenograft tumor bearing mice (tumor size: 150-200 mm³). The mice were sacrificed after 2 h of injection and the Fe³⁺ ions content of tissues (including heart, liver, spleen, lung, kidney, brain and tumors) were measured by inductively coupled plasma mass spectrometry (ICP-MS, 7500CE, Agilent, USA).

In Vivo Photothermal Therapy of tumor

The BALB/c mice were purchased from Beijing Vital River Laboratories Animal Technology Co. Ltd. and were taken care of under Institutional Animal Care and Use Committee of Xiamen University. 0.1 mL of 4T1 cell suspension (1×10⁵) was subcutaneously injected to the mice (6 - 8 week). When the tumor size was up to 150 - 200 mm³, the mice were intratumorally injected with or without 100 μL of Fe₃O₄-MTX@HBc NPs (12 mg·kg⁻¹).

For in vivo PTT, the tumors were irradiated by 808 nm NIR laser (1.5 W·cm⁻², facula 1.0 cm) for 5 min. The real-time temperature and infrared thermal images of the tumor sites were recorded by an infrared thermal camera (FLIR A35, FLIR, Sweden). For in vivo therapeutic efficacy, the growth rate of tumors was investigated by monitoring the tumor volumes (as calculated by the formula (1)) and body weight of mice per day.

\[ V = a \times b^2 / 2 \] (1)

Where a and b indicated the longest diameter and the shortest diameter, respectively. The tumors were photographed during the therapy period. The mice were euthanized on 10th day to detach the tissues (including heart, liver, spleen, lung, kidneys and tumors) and followed by further histological examinations using the hematoxylin and eosin (H&E) staining.

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Results and Discussion

Preparation of Fe₃O₄-MTX NPs

To facilitate the efficient encapsulation in HBc₁₄₄-His VLPs, monodispersed Fe₃O₄ NPs with a size of 10 - 15 nm were prepared (Fig. S1A). XRD patterns demonstrated the successful synthesis of Fe₃O₄ (Fig. S1B). For combination of PTT with chemotherapy, MTX was conjugated to the surface of Fe₃O₄ NPs via the covalent attachment between MTX and DMSA functionalized Fe₃O₄ NPs. FT-IR spectra were collected to prove the existence of amido bond (Fig. S2A). The magnetic property of MTX modified Fe₃O₄ NPs was measured and then displayed by magnetic hysteresis loop (Fig. S2B). The measurement showed that Fe₃O₄-MTX NPs owned a saturation magnetization value (Ms) of 9.1 emu g⁻¹. The drug loading efficiency of MTX in Fe₃O₄-MTX NPs was determined at 3.4%.

Preparation of Fe₃O₄-MTX@HBc NPs

In order to efficiently encapsulate the Fe₃O₄-MTX NPs into VLPs, HBc₁₄₄-His VLPs were engineered interiorly possessing histidine tags by genetic engineering to attract the Fe₃O₄-MTX-Ni²⁺ chelate during the process of encapsidation.²⁹ The HBc₁₄₄-His VLPs were obtained from E.coli. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to yield a single band corresponding to molecular weight of 14 kDa, indicating the corresponding molecular weight (14 KDa) of HBc₁₄₄-His subunit (Fig. 1A). To increase the dispersibility and biocompatibility of Fe₃O₄-MTX NPs under biological conditions, monodispersed Fe₃O₄-MTX@HBc NPs were prepared by encapsulating Fe₃O₄-MTX NPs into HBc₁₄₄-His VLPs. Morphology of Fe₃O₄-MTX@HBc NPs was confirmed by TEM image (Fig. 1B). The shells were formed regularly by the composition of HBc₁₄₄-His units. The Fe₃O₄-MTX cores inside the NPs presented much darker color than the protein shells. The analogous core-shell structure of Fe₃O₄@HBc VLPs has been demonstrated by atom force microscopy (AFM) and magnetic force microscopy (MFM) in our previous work.²⁸ The encapsulation efficiency of Fe₃O₄-MTX@HBc₁₄₄-His NPs could reach 89 % through counting more than 200 NPs according to the large-scale TEM images. The hydrodynamic size of Fe₃O₄-MTX@HBc NPs was 38.2 ± 5.6 nm measured by DLS (Fig. 1B). Hence, the encapsulation of Fe₃O₄-MTX NPs into HBc₁₄₄-His VLPs could yield monodispersed, well-defined, hybrid nanostructures and facilitate addressable functionalization of the Fe₃O₄-MTX NPs.

Photothermal effects

In order to confirm that Fe₃O₄-MTX@HBc NPs could be utilized as PTT agent, the photothermal performance was investigated. Fe₃O₄-MTX@HBc NPs in different concentrations were exposed to 808 nm NIR laser with power density of 1.5 W·cm⁻². When 0.1 mg·ml⁻¹ of Fe₃O₄-MTX@HBc NPs were exposed to NIR laser, the temperature of the suspension had an increase of 20.2 °C in 5 min. Analogously, when the concentration of Fe₃O₄-MTX@HBc NPs reached to 0.2 and 0.4 mg·ml⁻¹, the temperature of dispersions remarkably heightened by 27.5 °C and 33.3 °C, respectively. In comparison, the temperature of PBS as control did not change obviously in 5 min (Fig. 1C). Photothermal
stability of Fe₃O₄-MTX@HBc NPs was also investigated by 5 min of continuous irradiation followed by another 5 min of irradiation after dropping to the room temperature for three cycles. It can be visualized from the curves that there was no obvious photothermal ability change happened in 3 cycles (Fig. 1D). After NIR laser irradiation, Fe₃O₄-MTX@HBc NPs still exhibited good dispersion in PBS, indicating that Fe₃O₄-MTX@HBc NPs have an excellent photothermal stability and great potential for use in thermal therapy.

**In Vitro Photothermal-Chemo Therapy of Fe₃O₄-MTX@HBc NPs**

To investigate the *in vitro* photothermal effect of Fe₃O₄-MTX@HBc NPs, 4T1 cells were treated by Fe₃O₄-MTX@HBc NPs and NIR laser. The thermal images of 4T1 cell pellet were shown in Fig. 2A and the curves of cell pellet temperature versus time were shown in Fig. 2B. The temperature for cells treated by Fe₃O₄-MTX@HBc NPs increased to 50.6 °C, which was much higher than that for untreated cells (36.1 °C). To evaluate and compare the *in vitro* photothermal-chemo cytotoxicity of Fe₃O₄-MTX@HBc NPs with or without NIR light irradiation, the viability of 4T1 cells was determined by MTT assay. Various concentrations of Fe₃O₄-MTX@HBc NPs were incubated with 4T1 cells for 24 h and treated with or without NIR light irradiation (808 nm, 2W·cm⁻² for 5 min). As in shown Fig. 2C, Fe₃O₄-MTX@HBc NPs exhibited cytotoxicity without NIR light irradiation and all experiments evidenced an increasing cytotoxicity against 4T1 cells in a dose-dependent manner, revealing that the attachment of MTX to Fe₃O₄ NPs did not influence the chemo cytotoxicity of MTX. It presented a much higher efficacy of Fe₃O₄-MTX@HBc NPs to kill the tumor cells with 5 min of NIR irradiation, compared with the groups without light irradiation at all concentrations (Fig. 2C), indicating that Fe₃O₄-MTX@HBc NPs can effectively improve the efficiency of killing tumor cells *in vitro* by combining PTT and chemotherapy.

The cytotoxicity of Fe₃O₄-MTX@HBc NPs with NIR irradiation was verified by Annexin V-FITC/PI staining. Only untreated cells could not produce significant cell death. For the Fe₃O₄-MTX@HBc NPs groups, the cells with NIR irradiation exhibited significantly more apoptosis and necrosis (80.8%) than the cells without NIR irradiation (36.2%) (Fig. 2D). This result also confirmed the excellent synergistic effect of photothermal-chemo therapy of Fe₃O₄-MTX@HBc NPs.

**T2-weighted MRI capability**

As a noninvasive imaging tool, MRI is now widely used in hospitals for disease diagnosis, and can provide a three-dimensional anatomical image without exposing the body to ionizing radiation.³⁰ Fe₃O₄ NPs are commonly used as MRI contrast agents because they have the ability to shorten the T₂ relaxation time of surrounding water.³¹

![Figure 2](http://www.ntno.org)

**Figure 2.** The *in vitro* PTT of Fe₃O₄-MTX@HBc NPs for 4T1 cells. (A) Thermal images of untreated 4T1 cells (a) and 4T1 cells incubated with Fe₃O₄-MTX@HBc NPs (b) under laser irradiation (2 W·cm⁻², facula 0.5 cm) for 5 min; (B) The curves of cell pellet temperature versus time during irradiation; (C) Cell viability of 4T1 cells incubated with different concentrations of Fe₃O₄-MTX@HBc NPs and irradiated with or without NIR light (n = 3, ***p < 0.001); (D) Flow cytometry plots for cellular apoptosis and necrosis after different treatments.
To measure the T2-weighted MRI ability of Fe3O4-MTX@HBc NPs, the MRI of the NPs were performed. It can be observed from Fig. 3A that the brightness of Fe3O4-MTX@HBc suspensions were getting lower along with the increasing concentration in the T2-weighted MR images. In the meantime, the inverse relaxation time (T2-1) versus Fe concentration fitted line well and the r2 relativity values was 156 (mg·mL⁻¹)⁻¹ s⁻¹, indicating the potential of Fe3O4-MTX@HBc NPs to be a good T2-weighted MRI contrast agent. This might be due to that HBc VLPs could provide the advantages of proper size and shape, which can increase the chance of improving the rate of relaxation rates and the ideal modification of the contrast agent label.32

In vitro T2-weighted MRI was performed to compare the cellular uptake efficiency of Fe3O4-MTX@HBc and Fe3O4-MTX NPs (Fig. 3B). Comparing with the Fe3O4-MTX NPs treated group, the decrease of T2-weighted MR signal intensity and shorter T2 relaxation time were obtained after cells incubating with Fe3O4-MTX@HBc NPs for various durations. The results further demonstrated the higher cellular uptake efficiency of Fe3O4-MTX@HBc over Fe3O4-MTX NPs. This is due to that HBc144-His VLPs are a kind of biocompatible protein materials and can improve the dispersion and stability of Fe3O4-MTX under physiological conditions.

As the salient features of Fe3O4-MTX@HBc NPs, the magnetism was further evaluated in vivo on T2-weighted MRI to afford guidance for cancer therapy. As shown in Fig. 3C, the clear tumor boundary and much darker interior could be observed after injected of Fe3O4-MTX@HBc NPs. Which resulted from the decrease of T2-weighted MR signal intensity at tumor sites. Such an obvious difference in T2-weighted signal could help to find the position of tumor precisely, even the position of high-density of cancer cells. These results demonstrated that Fe3O4-MTX@HBc NPs could be used as a reliable MRI contrast agent for guiding therapy and reveal the in vivo biodistribution of themselves.

**In Vivo Biodistribution analysis of Fe3O4-MTX@HBc NPs**

The in vivo biodistribution of Fe3O4-MTX@HBc and Fe3O4-MTX NPs were evaluated by the Fe3⁺ ions content in tissues and tumors. As shown in Fig. S3, after intratumoral injecting nanoparticles for 2 h in mice, the tumor accumulation of Fe3O4-MTX@HBc NPs showed an enhancement of 1.39 folds than Fe3O4-MTX NPs treated group. These results were attributed to that the presence of HBc144-His VLP shell could increase the chemical stability and the dispersion of hydrophobic Fe3O4-MTX under physiological conditions, as well as could also provide a protective layer for drug molecules and magnetite nanoparticles from the recognition by the reticuloendothelial system, therefore allowing drugs to be accumulated in the tumor over the prolonged periods of time.

The mice in Fe3O4-MTX@HBc NPs treated groups (especially the one combined with PTT) exhibited remarkable delay in tumor growth or tumor regression compared to untreated group (Fig. 4C, D), demonstrating the outstanding photothermal-chemo synergistic effect of Fe3O4-MTX@HBc NPs. No obvious weight changing was observed for all groups (Fig. 4E). All the mice were alive during the 10 days of therapy period.
Figure 4. The PTT effect of Fe₃O₄-MTX@HBC in vivo. (A) Images and (B) the temperature curves of the tumor-bearing mice injected with or without Fe₃O₄-MTX@HBC under irradiation of 808 nm laser (1.5 W cm⁻², facula 1.0 cm); (C) Photos of the tumor-bearing mice, (D) change of body weight and (E) tumor volume for different treated groups during therapy period.

Figure 5. H&E stained images of tissues including heart, liver, spleen, lung, kidney and tumor after therapy. The scale bar is 100 μm.

In vivo toxicity of Fe₃O₄-MTX@HBC NPs was analyzed by H&E staining and no obvious organ damages or toxic side effects were discovered in all groups, meanwhile the apparent coagulative necrosis extensively existed in tumors in the mice of Fe₃O₄-MTX@HBC NPs treated groups (Fig. 5). All the results indicated that Fe₃O₄-MTX@HBC NPs had strong antitumorous effect in the photothermal-chemo
synergistic treatment process in vivo and could be used as remarkable nanotheranostic agent for further biomedical researches and applications.

Conclusions

In this work, we developed a novel kind of multifunctional core-shell nanoparticles, which encapsulated Fe₃O₄-MTX NPs in HBc144-His VLPs for MRI-guided photothermal-chemo therapy of cancer. The designed Fe₃O₄-MTX@HBc NPs possess good monodispersity, well-defined morphology, high r₂ relaxivity, good biocompatibility/biodegradation and strong NIR absorption feature. The outstanding MRI ability of Fe₃O₄-MTX@HBc NPs was confirmed on animals and visualized explicit effect of tumor magnetic resonance imaging. The therapeutic results of animal experiments reveal that the Fe₃O₄-MTX@HBc NPs present remarkable antitumor effect in combination of PTT and chemotherapy on cancer study. These excellent properties afford its applications as a theranostic nanoprobe for MRI-guided photothermal-chemo therapy of cancer.

Supplementary Material

Supplementary figures.

http://www.ntno.org/v02p0087s1.pdf

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Competing Interests

The authors have declared that no competing interest exists.

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