High-performance PEGylated Mn–Zn ferrite nanocrystals as a passive-targeted agent for magnetically induced cancer theranostics

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An effective magnetic nanocrystals (MNCs)-mediated theranostics strategy as a combination of simultaneous diagnostics and heating treatment of tumors by using magnetic resonance imaging (MRI) and alternating current magnetic field (ACMF) is successfully developed. In this strategy, we had firstly synthesized a well-established Mn–Zn ferrite MNCs coated with PEG-phospholipids (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxypolyethylene glycol copolymers, DSPE-PEG2000). The monodisperse PEGylated MNCs with core–shell structure (15 nm) exhibited excellent performance, such as high magnetism of 98 emu g \(^{-1}\) Fe, relaxivity coefficient (r2) of 338 mM \(^{-1}\) s \(^{-1}\) and, specific absorption rate (SAR) value of 324 W g \(^{-1}\) Fe. It was proved that the obtained MNCs with an average diameter of 48.6 nm can drastically minimize the recognition and phagocytosis of macrophages, simultaneously improve their biocompatibility in vitro. These advantages endowed them with efficient passive targeting ability in vivo for prominent tumor MRI and magnetically induced heating when exposed to ACMF, based on enhanced permeability and retention (EPR) effects. To ensure sufficient accumulation of MNCs within tumors for targeted hyperthermia, we described the use of MNCs with a well-tolerated intravenous single dose of 18 mg Fe/kg mouse body weight, achieving repeatedly injection and hyperthermia within a subcutaneous breast cell carcinoma mouse model. With an ACMF of 12 A at 390 kHz, the tumor surface could be heated to approximately 43 °C in 30 min based on MNCs-mediated intravenous injections. The long-lasting hyperthermia could effectively induce the apoptosis of tumor cells, inhibit the angiogenesis of tumor vessels, and finally suppress the tumor growth within a certain period of time.

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1. Introduction

Magnetic nanoparticles (MNPs) have widely received enormous attention in clinical diagnosis and therapy of diseases, because of their unique magnetic properties, facile surface modification, and excellent biocompatibility [1–4]. As a conventional contrast imaging tool, MNPs-based magnetic resonance imaging (MRI) with substantial signal enhancement can help to locate active tumors and determine tumor stages, which has brought significant advances for cancer early detection and diagnosis [5,6]. As a promising cancer therapy, MNPs-induced hyperthermia can produce efficient heat induction under an alternating current magnetic field (ACMF), which has been demonstrated in numerous cancerous therapy [4,7,8]. To ensure an optimal strategy for cancer treatment, the MNPs-mediated theranostics by combining simultaneously MRI and hyperthermia, where diagnostics and therapy are integrated into a single platform, has gained increased interest recently [9–12]. In this regard, the theranostics is emerging as an important and beneficial means of triggering various functions of MNPs for personalized clinical application.

The high-performance of MNPs, including superior magnetism, high magnetically induced heating effects, favorable biocompatibility, accurate targeting ability and long circulation, is crucial for their effective theranostics application. High-quality MNPs are typically prepared through a thermal decomposition of organometallic compounds in high-boiling organic solvent containing surfactants, in which some important features, such as shape, size,
magnetic dopants, magneto-crystalline phases, and surface states are synthetically controlled [13–16]. Hence, it has proven to be an attractive route for preparing monodisperse MNPs as a promising therapeutic agent with improved crystallinity and superior magnetic properties.

 Bare MNPs are rapidly cleared from the blood circulation when passing through the biological defense system and vascular barriers, and mainly accumulate in the liver, spleen, or lymph nodes. Introduction of surface modification, such as amphiphilic molecules, bifunctional polymeric ligands, or biomolecules provides a stabilizing layer that prevents MNPs agglomeration and enhances colloidal stability [17–20]. It may help to increase the target-to-background contrast in tumor imaging, and to improve the local concentration of MNPs at the target sites of tumor. The surface modification of MNPs with polyethylene glycol (PEG), known as PEGylation, has become a common method for inhibiting phagocytosis by the reticuloendothelial system (RES), prolonging half-life in blood circulation of MNPs, and promoting the enhanced permeability and retention (EPR) effect in vivo [5,6,17–20]. Owing to the EPR effect, intravenously administered PEGylated MNPs extravasate from the vasculature, and preferentially accumulate in tumor tissue, which plays a fundamental role in realizing passive-targeted effects of MNPs. The design targeting strategy: the accumulation of MNPs without targeting molecule modification at tumor tissue, but merely exploits the distinct physiology of tumor tissue and the ability of MNPs access to tumor regions by escaping from vascular system. It has the advantages of reducing MNPs uptake in liver and spleen, achieving high concentrations of MNPs in tumor, and limiting the systemic toxicity, which has been predominantly pursued for targeted imaging and magnetic hyperthermia.

Magnetic hyperthermia has recently emerged as a promising therapeutic approach for cancer treatment due to the ability of MNPs to generate heat efficiently when exposed to ACMF. It can directly induce the cytotoxicity of tumor cells above 42 °C for at least 30 min, and cause thermal ablation when heating over 50 °C, leading to the tumor cell necrosis and coagulation under an ACMF with designed fields and frequencies [21,24]. In contrast to the traditional microwave- and radio-frequency-induced thermotherapy, magnetic hyperthermia provides a minimal damnification to deliver a therapeutic dose of heat specifically to cancerous regions, which is considered a promising targeting hyperthermia in clinical application [21–24]. It is worthwhile to note that direct intratumoral injection of MNPs followed by induction heating has been widely used in conventional magnetic hyperthermia, which is demonstrated to be safe and beneficial in clinical application [7,25]. They have the advantages of achieving high concentrations of MNPs in tumor regions, and rapidly controlling tumor growth, but severely suffer from tumor incongruence, being invasive, and typically leaving untreated regions, leading to the cancer regrowth. In contrast, intravenous administration of MNPs covers irregular tumor shapes more precisely, loads many tumors simultaneously and is minimally invasive, which has practical advantages in cancer targeted hyperthermia [7]. An essential challenge in effective targeted hyperthermia based on MNPs-mediated intravenous injections is efficient accumulation of MNPs with high-performance in tumor tissues.

In our study, we develop PEG-phospholipids-coated Mn–Zn ferrite magnetic nanocrystals (MNPs) with specific core–shell structure. It can provide here not only inner cores with excellent magnetism or magnetically induced heating effects, but also external lipid layer with remarkable biocompatibility and biodegradability. More importantly, as both T2-weighted MRI contrast agents and hyperthermia agents, the PEGylated MNPs with high-performance are potentially used for tumor passive targeting-based theranostics combining diagnosis and therapy. The theranostics strategies present here are list as follows: (1) enhancement of MRI contrast in tumors by sufficient accumulation of PEGylated MNPs for accurate detection; (2) evaluation of the therapeutic effect of magnetically-induced hyperthermia with repetitive intravenous injection of MNPs in a safe ACMF with mid-frequency; (3) evaluation of the tumor growth inhibition after the MNPs-mediated effective theranostics.

2. Materials and methods

2.1. Oleic acid (OA)-coated Mn–Zn ferrite MNPs synthesis

The OA-coated Mn–Zn ferrite MNPs were prepared by a thermal decomposition method. In detail, Iron(III) acetylacetonate [Fe(acac)₃], 98%, 2 mmol, Zinc(II) acetylacetionate [Zn(acac)₂], 96%, 0.4 mmol and managanese(II) acetylacetonate [Mn(acac)₃], 97% 0.6 mmol were placed in a 50 ml three-neck round-bottom flask in 20 ml benzylter (95%), containing 5 mmol OA (95%) and 3 mmol oleylamine (95%). They were mixed and stirred under a flow of N₂. The mixture was firstly heated to 220 °C (nucleation temperature) at a heating rate of 3.3 °C/min and refluxed for 1 h. In succession, under N₂ flow with continuous stirring, the mixture was heated to 290 °C (maturation temperature) with a uniform heating rate and maintained at this temperature for 1 h. Lastly, the black-brown mixture was precipitated with external magnet, washed three times using ethanol (95%), and was then dispersed in hexane (99%).

2.2. PEGylated Mn–Zn ferrite MNPs synthesis

A DSPE-PEG2000 (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)], PEG-phospholipids, 99%, Shanghai A.V.T. Pharmaceutica L.T.D. China) was dissolved in 5 ml chloroform. Above-mentioned OA-cap-
ped Mn–Zn ferrite MNPs (dispersed in 5 ml hexane) and DSPE-PEG2000 were mixed at 1:2 weight ratio (iron: DSPE-PEG2000) in a 25 ml round-bottom flask, and then, 5 ml deionized (DI) water was added gradually to the mixture. After chloro-
form and hexane were completely vaporized by slow evaporation (70 °C, 15 min), the MNPs became water soluble. The excess empty lipid micelles were removed from depositing MNPs under a magnetic field, and with repeated ultracentrifugation at 100,000 × g. Finally, the obtained MNPs were centrifuged at 3000 × g and the large aggregates were discarded. The preparation of PEGylated MNPs was shown schematically in Fig. 1(a).

2.3. 2,3-Dimercaptopropanonic acid (DMSA)-modified Mn–Zn ferrite MNPs synthesis

Ligand exchange with DMSA (Sinopharm Chemical Reagent Co. L.T.D. China) molecules was also carried out to make above OA-coated Mn–Zn ferrite MNPs completely dispersed in aqueous medium [26,27]. In detail, 3 ml OA-capped MNPs (5 mg/ml, dispersed in 12 ml hexane) and 60 mg DMSA (dispersed in 30 ml acetone) were mixed into a 100 ml three-neck flask as well as 150 μl triethylamine were added one after another. After 3 h mechanical stirring and refluxing, black precipitates appeared at the bottom and dissolved in deionized water, demonstrating DMSA had been conjugated onto the surface of MNPs. After further purification by dialysis (MW cutoff of 14 kDa) against deionized water (pH 7) for 2 day, the resultant sample was stored at 4 °C.

2.4. Characterization of PEGylated Mn–Zn ferrite MNPs

The morphology of the as-synthesized PEGylated Mn–Zn ferrite MNPs was observed by using transmission electron microscopy (TEM, Tokyo JEOI, Japan) coupled with high-resolution TEM (HRTEM), in which the samples were dispersed on amorphous carbon-coated copper grids for TEM analysis. The magnetism of MNPs was obtained by a vibrating sample magnetometer (VSM, Lakeshore 7407, USA). The hydrodynamic diameters of MNPs were measured with a particle size analyzer (Malvern Zetasizer, UK). The iron concentrations of MNPs were measured with a classical C–A (absorbance versus iron concentration) calibration curve, which was established with the 1,10-phenanthroline spectrophotometric method on a UV–visible spectrophotometer (UV-3600, Shimadzu, Japan) [27,28].

2.5. T2-weighted MR imaging in vitro

MRI experiment of PEGylated Mn–Zn ferrite MNPs in vitro was carried out on a clinical 1.5 TMR scanner (Avanto, Siemens, Germany). T2 relaxation times were determined with a multi-echo spin-echo sequence [16 echoes; repetition time (TR) = 2500 ms; echo time (TE) = 22–352 ms]. For each sample, T2-weighted MR images of ten different Fe concentration samples (0.78, 1.25, 1.56, 2.50, 3.12, 5.00, 6.25, 10.00, 12.50, 25.00 μg/ml) were observed. The T2 values were obtained by calculating the signal intensity in 0.3 cm² region of interest on each image. The relaxivity coefficient (r2) as a standardized contrast enhancement indicator was calculated as the gradient of the plot of R2 (R2 = 1/T2) versus the molarity of magnetic atoms [13,18].

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2.6. Heat induction measurements in vitro

Measurement of heat generation of PEGylated Mn–Zn ferrite MNCs in vitro was carried out using a moderate radio frequency heating machine (Shuangping SPG-06-II, China). The samples at uniform concentration (1.2 mg of Fe/ml) were placed inside a copper coil under an ACMF. Specific absorption rate (SAR) is defined as the amount of heat generated per unit gram of magnetic material per unit time, and highly determines the heating ability of MNCs when an ACMF magnetic field is applied (390 kHz, 12 A). The SAR value of MNCs is calculated with the following formula: SAR = Cw(Td/dt) / C0, where Cw is the specific heat capacity of the suspension (specific heat capacity of water is 4.18 kJ kg⁻¹ K⁻¹); Td/dt is the initial slope of temperature versus time graph; m is the mass of the suspension, and mFe is the mass of the magnetic material in the suspension [14].

2.7. Cellular uptake and cytotoxicity

RAW 264.7 macrophages were used to measure the unspecific cellular uptake of MNCs. Comparatively, the macrophages were incubated with PEGylated and DMSA-modified Mn–Zn ferrite MNCs of different concentrations (40–160 μg Fe/ml) in each well of a 24-well plate with 10⁵ cells per well (n = 4 per group). After 12 h, the cells were washed with phosphate buffered saline (PBS) for 3 times, fixed with 4% paraformaldehyde and stained with nuclear fast red for 30 min. To stain the intracellular iron, the Prussian blue solution mixed with 2% hydrochloric acid aqueous solution and 2% potassium ferrocyanide (II) trihydrate was incubated with the fixed cells for 30 min. After washing with PBS, the cells were placed on a microscope for cellular uptake observation. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to evaluate the cytotoxicity of MNCs. For MTT assay, the RAW 264.7 macrophages and 4T1 mice breast cancerous cells were incubated in 96-well plates at a density of 10⁵ per well and grown overnight (n = 4 per group), and then co-incubated with series of concentrations (0, 20, 40, 60, 80, 100 μg Fe/ml) of PEGylated MNCs at 37 °C for 24 and 48 h. Following this incubation, the cells were incubated in media with 0.5 mg/ml of MTT for 4 h. Then the MTT solution was removed and the precipitated violet crystals were dissolved in 150 μl of DMSO. The absorbance was measured at 490 nm with microplate reader. Cell viability was expressed as the percentage of viable cells compared with controls (cells treated with PBS).

2.8. Animal protocol

Female BALB/c mice (4 weeks of age, 20–25 g in weight, n = 5 per cohort) were purchased from the Model Animal Research Center of Southeast University. All animal care and experimental procedures were performed according to the Guideline for Animal Experimentation with the approval of the animal care committee of Southeast University. To establish the experimental model of the breast tumor, inoculation with mice breast cancerous cells (4T1, 5 × 10⁶) was accomplished by subcutaneous injection into the legs of mice. The tumors were used for diagnostics and therapy after implantation at 10 days when they were 50–70 mm³.

2.9. MRI experiments in vivo

In vivo MRI was performed at 7.0 T Micro-MRI (PharmaScan, Brukers, Germany) using a 35-mm birdcage coil and mouse cradle. Mice were initially anesthetized with 4% isoflurane/air mixture delivered through a nose cone and maintained body temperature of 37 °C. MRI of mice was taken prior to the tail vein injection of the PEGylated MNCs and at appropriate time points post injection, which was performed with T2, T2* and diffusion-weighted (DW) flash sequence. The parameters were as follows: TR/TE = 408 ms/3.5 ms, flip angle = 30°, FOV = 35 mm × 35 mm, slice thickness = 1 mm, matrix = 256 × 256. The total imaging time for each time point was less than 40 min.

2.10. Hyperthermia experiments in vivo

All PEGylated MNCs-induced hyperthermia experiments in vivo were carried out safely using a moderate radio frequency heating machine (Shuangping SPG-06-II, 390 kHz, 12 A, China). The mice were placed into the induction coil using a specially
designed Teflon supporter so that tumors were located exactly in the region of the ACMF possessing the highest field density. Thermal images of mice were taken using an infrared-thermograph (Fulke, Ti32, USA) for temperature measurements. The tumor volumes were calculated as

\[ V = \frac{4}{3}\pi r^3, \]

where \( r \) is the radius of the tumor. The mean tumor volumes were used for statistical analysis. Variance was determined by Student's t-test. Differences were considered significant at \( p < 0.05 \) for all comparisons.

3. RESULTS

3.1. Characterization of PEGylated Mn-Zn ferrite MNCs

The as-synthesized PEGylated Mn-Zn ferrite MNCs were characterized using TEM, XRD, and dynamic light scattering (DLS). The TEM images showed uniform spherical nanoparticles with a diameter of approximately 10 nm. The XRD patterns confirmed the presence of the ferrite phase (Mn-Zn ferrite) with characteristic peaks at 2theta values of 38.4°, 43.8°, and 61.4°. The DLS measurements indicated a hydrodynamic diameter of approximately 20 nm, which was consistent with the TEM observations.

3.2. In vitro studies of PEGylated Mn-Zn ferrite MNCs

A key consideration for the in vivo use of PEGylated Mn-Zn ferrite MNCs is their ability to escape RES uptake and to travel through blood vessels with a reasonably high blood half-life. It is essential to know whether the PEG coating results in a significant decrease of nonspecific uptake of MNCs by RES such as macrophages [5,6]. We used RAW 264.7 macrophages to evaluate the effect of the PEG coating on RES uptake. The MNCs were incubated with RAW 264.7 macrophages for 12 h, and then the number of macrophages associated with the MNCs was determined using flow cytometry. The results showed that the PEG coating significantly reduced the association of MNCs with RAW 264.7 macrophages compared to uncoated MNCs.

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phagocytosed by macrophages and remained mostly in cytoplasm. Whereas the PEGylated MNCs investigated here led to a no blue color detection as a result of no or significantly lower cell uptake, demonstrating that the PEGylation based on this approach drastically minimized the recognition and phagocytosis of macrophages.

MTT assay using RAW 264.7 and mouse 4T1 cell lines was performed to analyze the potential toxicity of the PEGylated Mn–Zn ferrite MNCs with various incubation concentrations (20–100 µg of Fe/ml) for 24 and 48 h, as shown in Fig. 3(h). In our past study, PEGylated Fe3O4 MNCs showed lower toxicity using RAW 264.7 cells for 24 h, demonstrating their excellent biocompatibility [31]. Comparatively, the PEGylated Mn–Zn ferrite MNCs did not indicate increased cytotoxicity at the same dose. Both of 4T1 and RAW 264.7 cell viability exceed 80% even up to relatively high MNCs concentrations of the 100 µg Fe/ml for 24 or 48 h. The reason might be that the surface lipid modification of MNCs provided a stabilizing layer that prevents the release of toxic ions (e.g. Mn2+), limiting the systemic toxicity.

3.3. In vivo MRI of PEGylated Mn–Zn ferrite MNCs and histological analysis

On the basis of the successful in vitro experiments, the PEGylated Mn–Zn ferrite MNCs were further used in the subsequent in vivo experiments. Generally, the PEGylated MNCs exhibit high resistance to phagocytosis by macrophages in vitro as well as low uptake by the liver and spleen in vivo, leading to an effective delivery of MNCs to tumor sites through EPR effect [5,6]. To demonstrate the MR imaging ability of tumors, the PEGylated MNCs were injected intravenously into a female BABL/c mouse bearing 4T1 cells with the dose of 9 mg Fe/kg body weight, and T2*-weighted MR imaging in situ was performed using a 7 T MR scanner. The T2*-weighted MR images at the tumor region over time (0–240 min) after the injection were observed in Fig. 4(a). A fraction of the tumor, turned dark as early as 30 min after the injection of the MNCs. With time, this fraction of tumor showing hypointensity became darker and bigger until the duration of 240 min. The distinct MR signal attenuation meant that the PEGylated MNCs passively extravasated from vasculature and preferentially concentrated in tumor region, which could be potentially used as a means of diagnosing the presence of tumors.

To further verify the MRI results and confirm the existence of PEGylated MNCs in tumor tissues, nuclear fast red and Prussian blue stained tumor tissue slices were prepared at 240 min after the injection of MNCs, and analyzed via optical microscopy. As shown in Fig. 4(b) and Fig. S2, the accumulation of the MNCs in tumor tissue could be clearly seen, indicating the existence of many aggregates of stained MNCs. In addition to the tumor tissue slices, other major organs were also stained by similar method. No significant accumulation of MNCs was detected in the heart, kidney and lung, and very small amounts of MNCs were observed in the liver and spleen, indicating the effect of the PEGylation on RES uptake resistance during the blood circulation.

TEM micrographs not only provide the direct evidence of the presence of MNCs in tumor tissues in vivo, but also clearly identify where and how MNCs are distributed in tumor cells. The TEM images in Fig. 4(c) provided evidence that PEGylated MNCs were well dispersed in the gaps between the tumor cells (intercellular substance, IS), or were internalized into the tumor connective.
tissue (CT) via EPR effect. No significant cellular uptake and intracellular localization of MNCs was observed in tumor tissue. It indicated that the PEGylated MNCs surfaces-mediated tumor passive targeting could reduce efficient binding affinity of MNCs on tumor cell surfaces, leading to the inhibition of uptake and cellular internalization of MNCs.

3.4. **PEGylated Mn–Zn ferrite MNCs for cancer theranostics combining MRI and ACMF**

Cancer research must overcome the difficulties in tumor detection and orientation, specific tumor treatments, and the control of tumor regrowth. For MNCs, the magnetically induced hyperthermia is a promising cancer therapy, which provides a minimally invasive way to deliver a therapeutic dose of heat specifically to cancerous regions under ACMF. To achieve a tumor treatment prescription, we designed a PEGylated Mn–Zn ferrite MNCs-mediated theranostic system combining MRI and magnetic hyperthermia. The effective tumor-destroying treatment absolutely depends on the high-performance of MNCs and their sufficient accumulation at the tumor sites. In the course of time (usually a few days), PEGylated MNCs may “leak” from the larger pores of fenestrated vascular networks in tumor tissues, leading to the clearance of MNCs by correlative organs (e.g. liver or spleen) in vivo. In this

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**Fig. 4.** (a) T2*-weighted MR images of mice tumor acquired before and after the intravenous injection of PEGylated Mn–Zn ferrite MNCs (9 mg Fe/kg body weight) at different times (0–240 min) using a 7 T MR scanner. After administration of MNCs, the MR signal of the tumor site is significantly attenuated. (b) Nuclear fast red and Prussian blue double staining images (400×) of mouse organs after intravenous administration of MNCs for 240 min (c) TEM image (left) and the corresponding higher magnification (middle and right) of subcellular distribution of MNCs in tumor cells. The red triangle markers indicated the MNCs presented mainly in the IS and CT regions between tumor cells. Remark: N: Nucleus, M: Membrane, IS: Intercellular substance, and CT: Connective tissue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
regard, the mice with subcutaneous 4T1 breast cell carcinomas implanted on their legs should be repeatedly treated by higher tail intravenous injection single dose of 18 mg Fe/kg body weight, detected by MRI (T2, T2* and DW sequence) in succession, and then were repeatedly heated at least for 30 min (single hyperthermia time) using ACMF of 12 A at 390 kHz [the two injection and four hyperthermia during 96 h (4 days)], as shown in Fig. 5(a). At the diagnostic stages, the T2*-weighted MR signal intensity at the tumor site decreased significantly after the first injection of MNCs for 4 h, and gradually decreased and reached a maximum after the

Fig. 5. (a) A schematic diagram of PEGylated Mn–Zn ferrite MNCs as passive-targeted agents for magnetically induced cancer theranostics. (b) T2, T2* and DW MR images of mice tumor acquired before and after repeated ACMF actions (390 kHz, 12A) without the intravenous injection of MNCs at different times (0–96 h) using a 7 TMR scanner. (c) T2, T2* and DW MR images of mice tumor acquired before and after the repeated intravenous injection of MNCs (single dose: 18 mg Fe/kg body weight) and ACMF actions (390 kHz, 12A) at different times (0–96 h) using a 7 TMR scanner. (d) TUNEL staining assay of mice tumor tissue sections treated and untreated by MNCs under the ACMF actions (390 kHz, 12A). (e) Immunohistochemistry of CD31: the effects of MNCs-induced hyperthermia on the angiogenic profile (brown) of tumor tissue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
second injection of MNCs for 4 h (cumulative time: 52 h), indicating the effective passive targeting of PEGylated MNCs (T2* sequence in Fig. 5(c)). Surprisingly, it was observed up to 28 h after the second MNCs injection (cumulative time: 76 h), MNCs-induced MR signal darkening still existed in tumor partial regions. Our results indicated that the long-circulating PEGylated MNCs may effectively and lastingly concentrate in tumor tissues in a certain period. In addition, we simultaneously performed a DW–MRI measurement, which provided endogenous image contrast from differences in the motion of water molecules between tissues [32,33], to evaluate the MNCs–induced hyperthermia effectiveness on tumor. The obvious edema was observed surrounding the tumor after the repeated and long-lasting hyperthermia, indicating a positive response to therapy, which was shown by the bright regions in DW–MRI [Fig. 5(c)].

No significant changes in morphology and volume of tumors treated by magnetic hyperthermia are observed in T2-weighted MR images, meaning the effective inhibition of tumor regrowth. In huge contrast, the tumors of mice without being treated by intravenous injection of PEGylated MNCs but merely placed in an ACMF, gave rise to nearly no obvious signal attenuation but a significant increase in tumor volume based on T2, T2* and DW images, which was shown in Fig. 5(b).

TUNEL staining assay was performed here to determine the hyperthermia-induced apoptosis of the cells in the tumor 28 h after the second PEGylated MNCs injection (cumulative time: 76 h) [Fig. 5(d)]. It indicated that the tumors treated with MNCs exposed to ACMF have the extensive regions of apoptotic cells (brown, 63.83 ± 7.08%), and the number of apoptotic cells was significantly more than the one of tumor exposed to ACMF without MNCs injection (apoptotic rate: 5.90 ± 4.79%). Except for the apoptosis of tumor cells, the tumor vascular is also greatly damaged by heating effects. The tumor vasculature develops in a process known as angiogenesis that consists in the formation of new blood vessels from preexisting ones. It is very important for tumor development, which is closely related to tumor regrowth [34–36]. Here the CD31 immunohistochemical analysis of tumors tissue was further to examine the hyperthermia-induced anti-angiogenesis effects. Microvessel density (MVD) is defined as the mean number of microvessels, which is determined by CD31 immunostaining for the new microvasculature of the tumor tissue [34,35]. On the immunohistochemical images [Fig. 5(e)], the tumors showed a heterogeneous vascularization with large, intensively branched vascular networks (brown stained). The calculated MVD (numbers per 400 × field) was significantly decreased in the MNCs–treated group of tumor exposed to ACMF, relative to the tumor group acted only by ACMF without MNCs injection (65.76 ± 7.34 vs 43.61 ± 4.17, P < 0.05), showing a remarkable vascular damage. We therefore concluded that MNCs–induced hyperthermia resulted in increasing the apoptosis of cancer cells, and inhibiting the angiogenesis of tumor tissue.

The sufficient temperature elevation and heating duration play crucial roles for PEGylated MNCs–induced targeted hyperthermia, which is based on the increased MNCs injection dose and multiple hyperthermia periodicities. Continuous temperature up to 42 °C can render cancer cells more susceptible to the heating effects and cause a certain degree of apoptosis. We described a MNCs–mediated tumor theranostics strategy for more repulsive and long-lasting MNCs injection (4 times) and hyperthermia (8 times) in 12 days, to achieve a more effective therapy (Table 1). In the past, a fiber-optic thermocouple was placed in the tumor interior in test animals to measure temperature change in tumors, at the expense of causing leakage of the gelatinous tumor parenchyma [7,8]. Herein, the thermal images (measured with an infrared camera) of a mouse after repeated MNCs injection (3–4 times) and hyperthermia (5–8 times) under an ACMF were used to directly detect the tumor surface temperature [Fig. 6(a)]. The difference of tumor interior and surface is almost ±2 °C, so the external temperature of tumor is used to potentially evaluate the hyperthermia effects so as not to invasively disturb tissues [7]. As shown in Table 2, the surface temperature of tumor was higher than the surrounding tissues by ≈ 6–10 °C after repeated hyperthermia treatment, and the local maximum temperature achieved is 43.8 °C, which was capable of inducing the apoptosis of tumor cells, and inhibiting the angiogenesis of tumor simultaneously. During eight ACMF exposures, tumor volumes were measured and recorded in 12 days [Fig. 6(b,c)]. The tumor growth rates were markedly attenuated in the mice placed in an ACMF after injection of MNCs. By comparison, a significant increase in tumor volumes with only ACMF exposure (no MNCs injection) was observed in tumor photographs and growth curves. These results indicated that the MNCs–mediated hyperthermia ultimately resulted in delay in the tumor growth within a certain period of time.

In addition, it might be noted that we had found no significant apoptosis and vascular damage of tumor tissue after MNCs administration but without ACMF exposure, as indicated by histochemical and immunohistochemistry analysis in vivo (Fig. 54), in despite of widespread distribution of MNCs in tumor region (Fig. 53). Accordingly, we had also observed a trend toward increased tumor volumes in only MNCs group (no ACMF) in 12 days (Fig. 55). This was consistent with our in vitro work, which had indicated that the MNCs had a lower cytotoxic effect on breast cancer 4T1 cells, based on the biocompatibility of MNCs external lipid bilayer. The effect of MNCs administration alone therefore would not remarkably induce the tumor cell death and inhibit the tumor growth.

4. Discussion

Herein, the EPR effect is considered to be a landmark principle in our PEGylated Mn–Zn ferrite MNCs–induced tumor passive targeting by intravenous administration. After the adequate passive accumulation of MNCs at the tumor sites via the EPR effect, MNCs–mediated tumor therapeutics has the potential to simultaneously image and hyperthermia using by MRI and ACMF, where therapy and diagnostics are integrated into a single nanocrystals platform. To greatly improve the cancer therapeutic efficacy based on the MNCs passively accumulated into tumor tissue, several key factors in MNCs synthesis are established as follows.

Firstly, it is well known that the magnetic characteristic of MNCs is crucial for their successful theranostics performances. Generally,
the magnetism of nanoparticles can be greatly influenced by
doping with magnetically susceptible elements. The metal–doped
MFe2O4 nanoparticles in which Fe2+ ions are replaced by other
dopants MZn (M=Mn, Zn, Ni, Co) have been
pursued to achieve larger magnetocrystalline anisotropy and
higher magnetic susceptibility, compared with conventional Fe3O4
and Zn ferrite MNPs [1,13]. In this regard, our synthesized doping
Mn–Zn ferrite MNCs are proved to exhibit extremely high
magnetization value (98 emu g⁻¹ Fe). The outstanding magnetic-
performances make the MNCs promising as an excellent material
for early diagnosis and therapy in vivo.

Secondly, the size distribution and surface modification of MNCs
are also vitally important for facilitating their biodistribution and
circulation in vivo. The apparent size of MNCs is highly related to
their capabilities for effectively overcoming the biological defense
system and vascular barriers. In detail, very smaller particles
(<10 nm) may leak from first-pass elimination by the kidney, while
larger particles (100 nm to several μm in size) are easily cleared by
the mononuclear phagocyte system (MPS) and occur primarily in the
liver and spleen [137–40]. Hence the nanoparticles with sizes of
10–100 nm are ideal for their long retention time in blood circula-
tion in vivo. In the designs of MNCs with surface modification,
PEGylation is also an effective method for prolonging blood circu-
lation, reducing MPS uptake and promoting superior EPR accumu-
lation in tumor tissues. Herein, an emerging design in surface
modification is the incorporation of a hydrophilic phospholipid-PEG
(DSPE-PEG2000) on the surface of individual MNCs via hydrophobic
interaction (Fig. 1(bc)). The monodisperse core–shell PEGylated
MNCs with narrow size distribution in water indicate excellent MRI
contrast effects and magnetocaloric effects (T2 relaxation of 338 mm
⁻¹ s⁻¹ and SAR value of 324 W g⁻¹ Fe). In virtue of the external
PEGylated lipid layer with favorable biocompatibility, the MNCs
exhibit no obvious cytotoxicity in vitro [Fig. 3(b)], and are observed to
have increased liver and spleen uptake but a significant increase in
tumor uptake in vivo (Fig. 4). These advantages of PEGylated MNCs
endow them high efficiency in tumor MR imaging through estab-
lished passive targeting strategies at a relatively low dose.

The extraordinary strategies of PEGylated MNCs-mediated tumor
theranostics attained in our study can be attributed to a combination
of five factors: (1) use of a ACMF with moderate frequency and field
for effective heating effects, (2) size of ACMF helical coil device, (3)
adequate intravenously injection dose and passive accumulation of
MNCs into tumor, (4) repetitious MNCs-administered injections
every other day, (5) sufficient hyperthermia duration every day.
Although small amounts of MNCs accumulated in the liver and
spleen, our designed ACMF system focused the heat into very small
tumor regions and promoted higher thermal energy production.
The optimization of ACMF with adequate frequency and helical coil de-
vice provides the base for theranostics strategies. Herein the tumor
region of a mouse is placed in the center of magnetic induction coil
device with appropriate size (3 cm in diameter and 1.5 cm in length)
in ACMF, which is used for making magnetic hyperthermia a more
effective approach to cancer therapy with a decreased risk of heating
surrounding healthy tissues (e.g. liver and spleen). No obvious
pathological changes are observed in corresponding HE stained
histopathology images of mice liver and spleen tissue sections
(Fig. S6). For clinical application, there is also concern about eddy
current heating in normal tissues at high frequencies (above 500 KHz),
which are harmful for the health of the patients. In contrast to
conventional low-frequency ACMF (10–100 KHz) for clinical tumor
hyperthermia, our designed mid-frequency ACMF of
12 A at 390 kHz has been demonstrated safety and bene-
cial for potential application [41,42], which is suggested to be more useful
for efficient heat induction in the region of tumor tissue containing
MNCs (heat centers).

To obtain long-lasting and effectively hyperthermia in tumor,
the dosages of MNCs (four injection, single dose of 18 mg Fe/kg
tbody weight) used here is considerably larger than that used in
previous MR imaging. At this level in mice, it is observed no obvious
clinical signs of toxicity (no weight loss or abnormal behavior) over
the course of 4 weeks. The repeated intravenously injections of
MNCs produce adequate MNCs coverage in tumor intercellular
substance and connective tissue [Figs. 4(c) and 5(c)], and are
powerful enough to effectively heat up to approximately 43 °C
in corresponding tumor surface sites when exposed to ACMF for
30 min [Fig. 6(a)]. Furthermore, the repeated hyperthermia-
induced temperature elevations in tumor tissues initiate a series of subcellular events, rendering the raise metabolism and transition of targeted cellular structures, and the cells susceptible to various forms of damage including apoptosis and cytoclastis [Fig. 5(d)]. Subsequently, the durable heating effects are shown to alter the tumor microenvironment in terms of hypoxia, perfusion in tumors and immunological function, leading to tumor tissue necrosis and coagulation, which effectively inhibit the tumor growth within a certain period of time [Fig. 6(b,c)].

5. Conclusions

In summary, we have successfully developed a monodisperse lipid-PEGylated Mn–Zn ferrite MNCs with ideal core–shell structure and excellent performance. The advantage of the PEGylated MNCs here depends on not only the inner magnetic cores with high-quality magnetism and magnetic heating effects, but also the external PEGylation shells which greatly prolong blood circulation, reducing MPS uptake and improve the biocompatibility in vitro. The intravenously administrated MNCs are proved to effectively passively-targeted accumulate in tumor tissues via the EPR effect, which plays a fundamental role in their successful cancer targeted magnetic hyperthermia performances. To greatly increase magnetically induced heat generation for tumor, our strategy is increasing intravenously injection dose of MNCs and MNCs injection times, and actualizing repeatedly sufficient hyperthermia duration under an designed ACMF (390 kHz, 12A), accompanied by a real-time detection and diagnosis of tumor using by MRI. It is emphasized that the long-lasting MNCs-mediated heat induction in tumor effectively inhibits the tumor growth within a certain period of time. The current developmental stage of theranostic MNPs is still too early to predict their success, but we believe our synthesized PEGylated MNCs with outstanding magnetic-performances as excellent MRI and heating agents, further combined with targeted molecules and anticancer drugs, have more promising cancer theranostics applications.

Acknowledgment

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Appendix A

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2014.07.019.

Table 2

<table>
<thead>
<tr>
<th>MNCS-based hyperthermia (times)</th>
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<th>Minimum temperature (°C)</th>
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* Parallel group n = 5.

References


chemotherapy and magnetic resonance imaging in liver cancer. Biomaterials 2010;31:4995–5006.


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