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ORIGINAL RESEARCH

Heating effect and biocompatibility of bacterial magnetosomes as potential materials used in magnetic fluid hyperthermia

Rui-ting Liu[a], Jie Liu[a,c], Jie-qiong Tong[a,c], Tao Tang[a,b], Wei-Chao Kong[a,c], Xiao-wen Wang[a], Ying Li[b,*], Jin-tian Tang[a,]**

[a]*Key Laboratory of Particle & Radiation Imaging (Tsinghua University), Ministry of Education, Department of Engineering Physics, Tsinghua University, Beijing 100084, China*
[b]*State Key Laboratories for Agrobiotechnology and College of Biological Sciences, China Agricultural University, Beijing 100083, China*
[c]*Department of Pharmaceutics, Beijing University of Chinese Medicine, Beijing 100102, China*

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Properties;
Biocompatibility

Abstract Magnetic fluid hyperthermia (MFH) promises to be a viable alternative in the treatment of localized cancerous tumors. The treatment consists of introducing nanoparticles as energy absorbent agents in tumor tissue under an oscillating magnetic field, where nanoparticles dissipate energy in the form of heat, causing a localized rise in the temperature and tumor cell death. Traditional magnetic fluid under study is artificial magnetic nanoparticles. This work seeks to introduce the new natural biologic magnetic material bacterial magnetosomes (BMs) to be used in MFH. Properties of magnetosomes and chemically synthesized magnetic nanoparticles (MNPs), such as morphology, magnetic properties and their heating effects under magnetic field were compared. Cytotoxicity studies using human breast cancer cells MCF-7 indicated that cell viability

*Corresponding author. Tel.: +86 10 62733751; fax: +86 10 62732012.
**Corresponding author. Tel./fax: +86 10 62796784.
E-mail addresses: yingli528@vip.sina.com (Y. Li), tangjt@mail.tsinghua.edu.cn (J.-t. Tang).

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could be significantly decreased by the heat derived from BMs and MNPs under alternative magnetic field. Biocompatibility of BMs and MNPs was compared in terms of evaluating their acute toxicity in mice and their decomposition abilities in vitro, and it showed that magnetosomes exhibit a lower toxicity. These findings provide evidence for beneficial activities of magnetosomes in MFH and support the continued investigation of it to be applied in biomedicine.

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1. Introduction

Currently, tumor extirpation, chemotherapy and radiotherapy are the three main methods to treat cancer. Although countless lives have been saved by these approaches, they are not always enough to eradicate the disease. Magnetic fluid hyperthermia (MFH) is one of the efforts that are being made in treating tumors to allow the patient a better quality of life. It involves injecting the fluid containing magnetic nanoparticles directly into tumors, and then the alternating magnetic field placed around the tumors will destroy them by heat dissipated by the nanoparticles. MFH could prevent unnecessary heating in healthy tissues because only the magnetic nanoparticles absorb the magnetic field energy [1].

Magnetic nanoparticles in MFH are the materials that absorb energy and turn it into heat, so they play a very import part in the therapy. The increase in temperature should depend on the magnetic properties of the material, the frequency of oscillation, the strength of the magnetic field and so on. Until recently, most of the magnetic fluid under study is artificial magnetic nanoparticles, mainly in the form of superparamagnetic iron oxide nanoparticles, such as amino silane iron oxide nanoparticles and applications of the magnetic materials as heating mediator for hyperthermia with the goal of tumor therapy have been studied in vitro, in vivo and in human trials, with success [2,3]. Nowadays, concerns have been raised regarding the toxicity induced by the presence of the chemically synthesized nanoparticles though significant progress has been made in MFH, so efforts are being made in order to optimize the features of nanoparticles.

Bacterial magnetosomes (BMs), which could be obtained by biomineralization process in the magnetotactic bacteria, consist of magnetic mineral crystals magnetite or greigite enveloped by biological membranes that contain phospholipids and specific proteins [4]. BMs have attracted much attention in the three decades since their unique feature making them could be considered used as a new natural biologic magnetic material in biomedicine. Studies have been performed to use BMs as carrier, such as proteins, nucleic acids, antibodies or drug [5–7], but have not been used as heating mediator in MFH. It was shown that comparing with artificial magnetic nanoparticles (MNPs), BMs exhibit better heating effects under the same magnetic field [8,9] and the lipid membrane on magnetosome surface endowed them with better biocomparibility.

Although magnetic properties of BMs have been evaluated [10] and it predicted that BMs could be considered as good materials for the biomedical applications in hyperthermia treatments [9], little has been done to compare the effects of MNPs and BMs in hyperthermia. In this article, the heating efficiency and antitumoral activity of BMs were evaluated, comparing with superparamagnetic chemically synthesized magnetic nanoparticles (MNPs), which are currently being tested for MFH [3], and hyperthermia was performed using hot water or the two kinds of particles. Biocompatibility of BMs and MNPs was also compared in the form of their acute toxicity in mice. The results showed that BMs possess a promising applicable prospect in the magnetic induction hyperthermia field for their special configuration.

2. Material and methods

2.1. Materials

BMs were isolated from *M. gryphiswaldense* MSR-1 based on the previously described method [11]. Spherical Fe_3O_4 MNPs with amino silane as the capping agent were synthesized by co-precipitation in our lab [12]. BMs and MNPs were sterilized by Co_{60} (15 kGy) before injection.

Male and Female Bal/c mice (18–22 g) purchased from Laboratory Animal Center of Tsinghua University were used to estimate the acute toxicity of the nanoparticles. These mice were housed with free access to standard food and water at a room temperature of 21 ± 2 °C, relative humidity of $45 \pm 15\%$ and a 12 h-light/dark cycle. All animal experiments were conducted in accordance with the National Institutes of Health Guide for the care and use of Laboratory animals and a protocol approved by the guidelines of the Chinese Society of Laboratory Animal Sciences. Every effort was made to minimize the suffering of the animals and the number of animals used.

2.2. Examination of physical properties of particles

The morphology of BMs and MNPs were viewed by electron microscope (TEM) H-800 (Brookhaven Instruments Corp., USA), and crystal-size distributions were quantified by measuring the average particle size. Vibrating sample magnetometer (VSM) was used to measure the magnetic properties of BMs and MNPs, and the measurements were carried out in the field region of ± 1 T at room temperature.

2.3. Heating effect of particles

Magnetic particles could absorb energy from the alternating magnetic field and converting it into heat. So, inductive heating property of the BMs and MNPs of a serious concentrations (diluted in PBS) were performed by exposing the particles under the alternative magnetic field (AMF) of frequency of 300 kHz and field amplitude of 110 Gs generated by inductive heating device (Shuangping Instrument Technology, Co., Ltd, Shenzhen, China). Thermal-couple temperature probe (Model

IT-18, copper-constantan, Physitemp, NJ, USA) was applied for the temperature measurement. The probe fibers were connected to a four-channel millivoltmeter (Model XSOL-4, Beijing Kunlun Tianchen Instrument Technology, Co., Ltd, Beijing, China) and the data of sample temperature were collected every 12 s by PC with home-written software.

2.4. Heating treatment by water bath

Human breast cancer cells MCF-7 and mouse fibroblasts (L-929) cells were cultured in 1640 medium supplemented with 10% fetal calf serum in 5% CO_2 humidified atmosphere at 37 °C. Cells were fed three times a week with fresh medium and passaged when 80% confluent. Cells were seeded at a concentration of 8000 cells/well in 96-well assay plates and grown for 1 day at 37 °C and 5% CO_2 before treatment. The next day, the plates were placed in hot water bath at a series of temperatures (45 °C, 47 °C, 50 °C) for a period of 30 min.

Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl- tetrazolium bromide (MTT) assay. In brief, culture media was added with MTT (final concentration: 0.5 mg/ml) at 37 °C in a 5% CO_2 incubator for 4 h, then the media was replaced with 150 µl dimethyl sulphoxide. After gentle stirring, the optical density (OD) of the samples was then read with a microplate reader for ELISA (UNICO, UV-2000; 540 nm). The inhibition ratio of every treated group was calculated using the formula: Inhibition ratio $=(OD_{\text{control}}-OD_{\text{treated}})/OD_{\text{control}} \times 100\%$.

2.5. Magnetic fluid hyperthermia

MCF-7 cells were cultured as described above. Cells were detached from the culturing flask by trypsinization, resuspended in culture media and counted. A concentration of 1×10^5 cells/well were seeded in 6.5 mm Transwell filter inserts (Millipore), which were placed in 24-well assay plates. The next day, 10 mg/ml BMs or 15 mg/ml MNPs (the concentrations were chosen according to the preliminary experiments) were added into the 24-well plates, and the cells were subjected to AMF of 300 kHz, 110 Gs generated by inductive heating device (Shuangping Instrument Technology, Co., Ltd, Shenzhen, China), and cells under the same condition but without applying with AMF were served as control. A circulator bath was used to maintain the ambient temperature surrounding the plates around 37 °C. Thermal-couple temperature probe was applied for the temperature measurement. Samples took an average of 2 min to reach the desired temperature, after which exposure time started. The next day, the cells' viabilities were analyzed using MTT assay.

2.6. Decomposition of nanoparticles analysis in vitro

To simulate intracellular lysosomal conditions, BMs and MNPs at a concentration of 0.1 mg/ml were suspended in a solution of 0.1 mg/ml crude protease from bovine pancreas (Sigma) in 1 ml of PBS solution at 37 °C under constant stirring. The solution of pH was adjusted by the titration of 1.0 M HCl and 1.0 M NaOH to achieve pH values of 5.6 (the pH condition in lysosomes in vivo). Following incubation for 1, 3, 7, 14 and 28 day, the nanoparticle suspensions were viewed under TEM.

2.7. Acute toxicity analysis in vivo

Nanoparticles were suspended in 0.9% sterile NaCl solution, and they were injected into the caudal vein of the mice ($n=10$/group, 5/sex; MNPs dosage: 135, 180, 240 mg/kg; BMs dosage: 270, 360, 480 mg/kg), and the group of control was treated with the same volume of saline (10 ml/kg). The doses were based on the preliminary tests. After the single treatment with nanoparticles, mice were observed frequently during the first 4 h; thereafter observed every 8 h and weighed every 24 h. If the animal died, the time of death was recorded, and the nature of adverse effects was noted. Dead animals were autopsied and examined macroscopically for any pathological changes. These mice were observed for 14 days before sacrificed, and blood samples were taken for routine examination, liver and kidney function tests. Hearts, livers, spleens, lungs, kidneys and brains were removed, weighed and sectioned for hematoxylin-eosin (HE) staining to observe underlying pathological changes.

2.8. Data analysis

Statistical analysis was conducted using SPSS (SPSS Inc., Chicago, IL). Results were compared among groups by one-way ANOVA followed by Dunnett's test. $P<0.05$ was assumed to indicate statistical significance.

3. Results

3.1. Analysis of particle morphology

TEM images of BMs and MNPs were presented in Fig. 1. We can see from Fig. 1a that cubo-octahedral BMs isolated from MSR-1 were dispersed very well and were often arranged in chains or sticking together. As it was reported before [4], lipid magnetosome membranes (MMs) were found surrounding every BM. Compared with BMs, MNPs were spherical and seemed more likely to aggregate.

We measured the dimensions of BMs and MNPs and found nearly 50% size of BMs distributed from 35 nm to 50 nm (mean size 40 nm), while the maximum of the size distribution range of MNPs was 6 nm to 15 nm, and had a mean size of 10 nm.

3.2. Analysis of magnetic properties

Fig. 2 shows the magnetization (M) versus field (H) curve at 300 K for the two samples. From the curves, we can see that BMs has a coercivity of 52.366 Oe and remnance of 34.140 emu/g, existing ferromagnetic behavior. Compared with BMs, the coercivity of MNPs was 4.0810 Oe, and the remnance was 65.227 emu/g, existing superparamagnetic behavior. The results showed that both BMs and MNPs had high magnetic response due to their perfect crystallinity.

3.3. Heating effect of particles

The heating profiles of BMs and MNPs in different concentrations under AMF of 300 kHz were shown in Fig. 3. As shown in the figures, higher particle concentrations resulted in

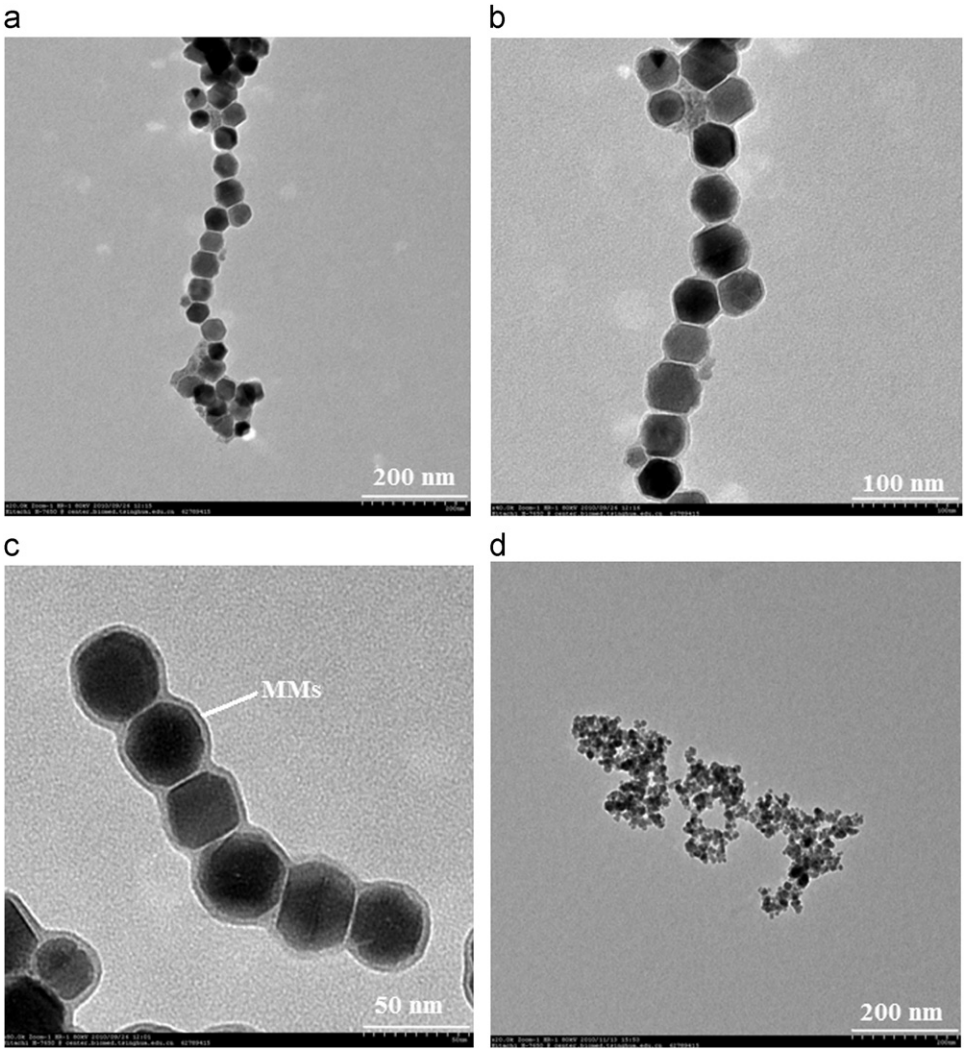

Fig. 1 TEM images of BMs (a, b, c) and MNPs (d). MMs stands for magnetosome membranes.

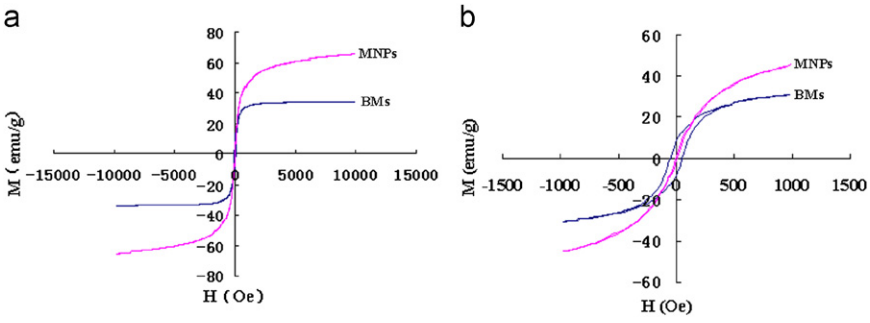

Fig. 2 Magnetization curve of BMs and MNPs obtained by VSM. (b) is an enlargement of (a). From the curves we can see that BMs show ferromagnetic behavior while MNPs show superparamagnetic behavior.

greater temperature increasing. And compare the two kinds of nanoparticles, the heating effects of BMs seemed better than that of MNPs. Take 47 °C for example, BMs needed 1.3, 0.7 and 0.4 min to reach the temperature, in the concentration of 10, 15 and 20 mg/ml, respectively, while the time for MNPs in the same concentration was 1.9, 1.5 and 0.7 min.

3.4. Heating treatment by water bath in breast cancer cells

Hot water hyperthermia were performed at three temperatures, 45 °C, 47 °C and 50 °C, with an exposure period of 30 min. Inhibitory rates of hot water were analyzed 24 h after application. As we can see in Fig. 4a, when it was 45 °C, the

inhibitory rate was 58.7% to MCF-7 cells and 42.7% to L929 cells. The thermal dose of 47 °C exhibited an inhibitory rate of 62.2% and 53.2% to MCF-7 and L929 cells, respectively. When the temperature reached 50 °C, 69.9% of the L929 cells and 82.3% of the MCF-7 cells died, indicating tumor cells are more sensitive to heat treatment.

3.5. *Magnetic fluid hyperthermia*

MFH was performed by using BMs or MNPs suspensions. The temperature of the media was continuously monitored during magnetic field application with a flame couple. With the purpose of comparing hot water hyperthermia with that resulting from energy dissipation from magnetic nanoparticles, the intermediate thermal dose of 47 °C was applied by controlling the intensity of the magnetic field. It was found that the magnetic field intensity of 138 Gs was needed to generate the thermal dose of 47 °C.

Both of the cell inhibitory rates were around 80% when using BMs and MNPs under AMF (Fig. 4b). Compare with that of ~60% in hot water hyperthermia under the same thermal dose, magnetic fluid hyperthermia induced a more significant reduction in cell viability. It could be seen that the viability of cells remained high in the absence of AMF, indicating the viability reduction was due to the AMF. In addition, compared with MNPs, lower concentration of BMs was needed to generate the thermal dose, indicating that BMs have better heating effect than MNPs.

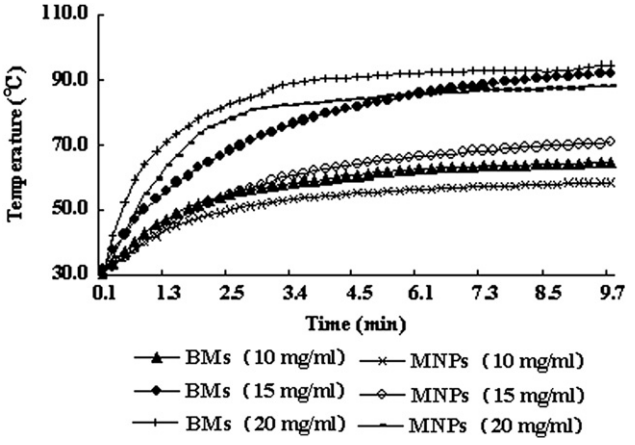

Fig. 3 Variation of temperature of suspension containing BMs or MNPs of different concentrations. The suspension was exposed to an AMF frequency of 300 kHz and field amplitude of 110 Gs.

3.6. *Decomposition of nanoparticles analysis in vitro*

Crude protease from bovine pancreas in 1 ml of PBS solution at pH of 5.6 was used to simulate intracellular lysosomal conditions, and BMs and MNPs of 0.1 mg/ml were incubated in the solution for 1, 3, 7, 14, 28 and 42 days with constant stirring at 37 °C. We can see from Fig. 5 that BMs seemed not intact and were surrounded by some cloud form in 28 day time, indicating BMs were decomposed. And when imaged in 42 day time, we could hardly found any BMs, just some of their remnants. While for MNPs, the morphology of them seemed have not changed much compared with new ones.

3.7. *Acute toxicity analysis in vivo*

Suspensions of nanoparticles were injected into the caudal vein of the mice, and the mortalities of mice injected with 135, 180 and 240 mg/kg MNPs were 30%, 50% and 67.70%, respectively. Most of the dead mice died during the 4 h after injection, but the surviving mice showed normal behavior and increased weight over the course of the experiment. In the groups of BMs-treated, all mice survived except one treated with the high dose of BMs (480 mg/kg). But some of the surviving mice injected with BMs showed slight listlessness and decreasing weight during the first four days, after which they recovered (Supplementary Fig. A.1).

The surviving mice were sacrificed in the 14th day after nanoparticles were injected. The major organs were removed and weighed. TEM examination of ultrathin sections from livers showed the presence of BMs and MNPs in liver cells. Endocytotic vesicles containing BMs were usually merged with lysosomes, but vesicles containing MNPs did not seem to merge with lysosomes (Fig. 6). It indicated that BMs and MNPs may undergo different decomposition in the liver. In addition, prussian blue staining was performed in the sections of major organs of mice to study the nanoparticle distribution. In both MNPs and BMs mice, sporadic blue particles were seen in the livers and spleens (Supplementary Fig. A.2). The results of organ coefficients in the mice, liver and kidney

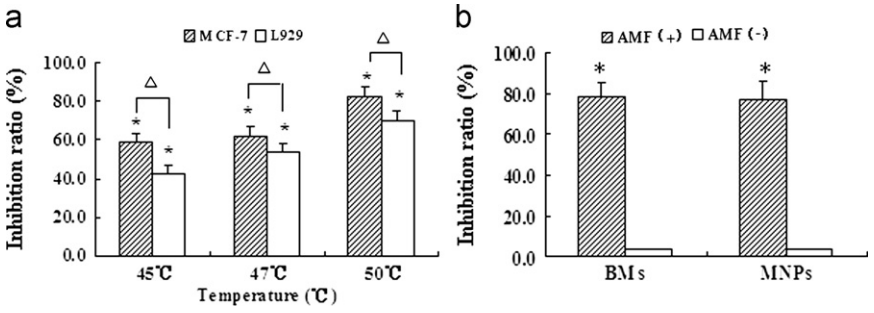

Fig. 4 Viability analysis of cells exposed to various modes of hyperthermia. (a) MCF-7 cells and L929 cells were exposed to hot water hyperthermia in thermal doses of 45 °C, 47 °C and 50 °C. (b) MCF-7 cells were exposed to MFH using BMs or MNPs in the thermal dose of 47 °C. The inhibition ratio was detected by MTT assay. Data are expressed as mean ± S.E.M. Assays were repeated three times and each condition was tested in triplicate for each assay. $^{*}P<0.05$ control, $\Delta P<0.05$ between the two kinds of cells.

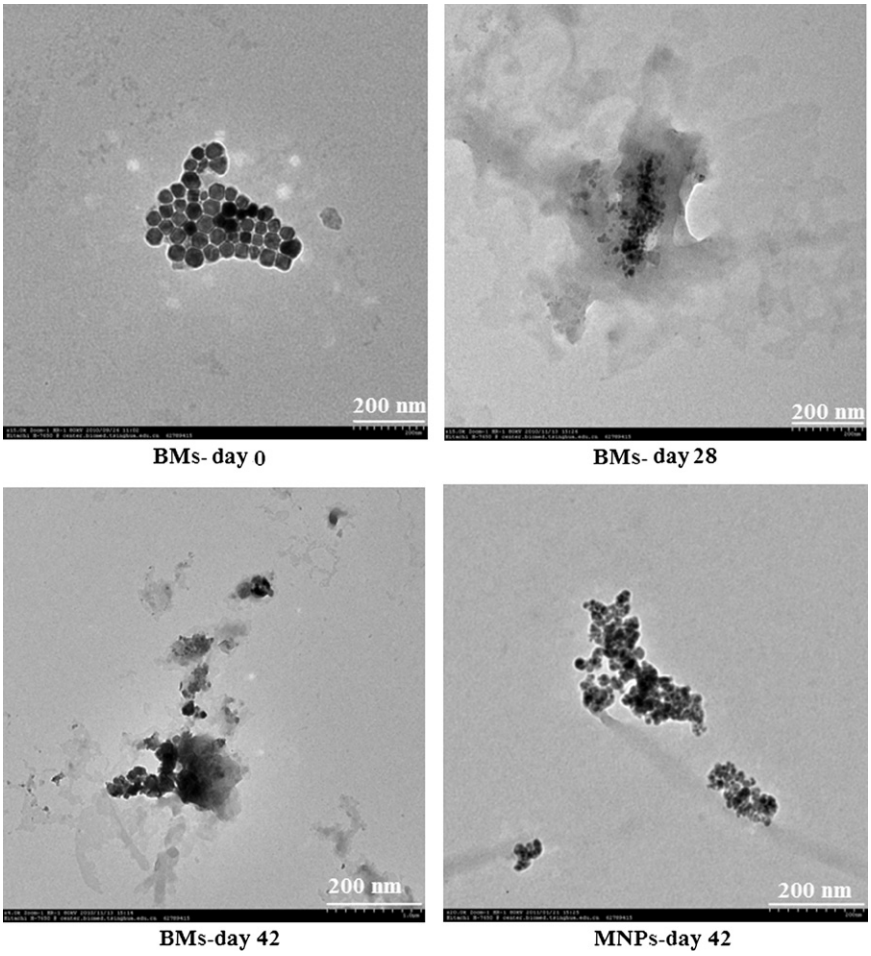

Fig. 5 Representative TEM images of BMS and MNPs decomposed by crude protease in vitro. It seems that BMs could hardly be found in 42 days time, while the morphology of MNPs have not changed too much.

function examinations and routine blood tests are shown in supplementary data.

4. Discussion

Ideal methods of cancer treatment would successfully achieve tumor ablation at all stages of the cancer disease in a noninvasive manner and with a minimum of side effects. In this view, thermotherapy consisting of heating tumors to death appears to offer a suitable method. Despite many advantages, development of thermotherapy is limited mainly because of the lack of tissue specificity. Metallic nanoparticles offered a breakthrough in the improvement of thermotherapy specificity to solve the difficulties related to targeting the irradiation area, giving a new chance of rapid clinical development of thermotherapy [13]. Actually, the iron oxide nanoparticle has been used for more than 10 years in MRI [14], and it seems a safe material for thermotherapy in clinical trails [3]. The unique physicochemical properties of nanoparticles are of importance for the biomedical uses. Generally, ideal heating material used in MFH should have the following characteristics: first, satisfactory heating effect under AMF; second, favorable physicochemical properties, including the magnetic characterization, crystal size, crystallinity, stability, dispersity and so on. Last but not the least, the material must have high biocompatibility, which means that it should be safe enough for medical use. BMs have been receiving attention since 1975 [15], and they were suggested as potential diagnostic and therapeutic tools recently [8]. As an exploratory study, the present work attempts to introduce the natural nanoparticle BMs to be used in the promising thermotherapy of tumors.

Increased heating ability of nanoparticles is one of the most important challenges in order to minimize the dosage of magnetic fluid needed to reach therapeutic temperatures in MFH. By exposing MNPs and BMs under the AMF of 300 kHz, we found that BMs exhibit a higher heating speed and temperature, which is consistent with the previous studies [9], so lower concentration of BMs was needed to generate the quantity of heat in MFH, as indicated in the present study. On the reasons for the increasing heating ability, we hypothesize that the different particle size could be one of the most important causes for it, as it has been predicted that there is an optimum particle size, which would yield the highest heating rate for a given set of measurement conditions [16]. So, it could be presumed that chemically synthesized nanoparitcles could potentially have the similar heating ability as BMs if they were enlarged to 40 nm. However, it has been proved that it has not yet reached the heating efficiency of BMs, even not as good as MNPs of

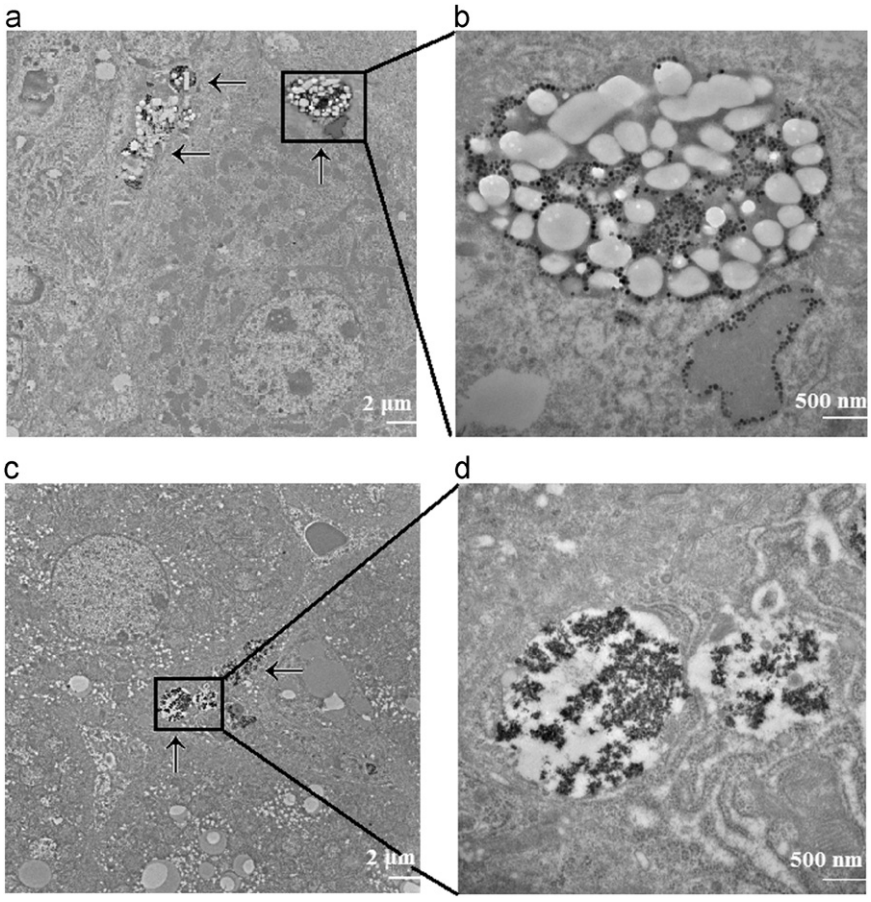

Fig. 6 Representative TEM images of livers in mice treated with BMs or MNPs. (a) Liver of mice injected with BMs, the arrows indicate BMs. (b) A partial enlarged image of BMs in the liver. (c) Liver of mice injected with MNPs, the arrows indicate MNPs. (d) A partial enlarged image of MNPs in the liver.

10 nm [16], probably because of the difficulty to chemically synthesize monodomain, well-crystallized ferromagnetic nanoparticles with good stability. Secondly, the different magnetic properties of BMs and MNPs could be another reason. Compared with MNPs modified with amino silane, we can see that both of the coercivity and area of the hysteresis loops of BMs increased, so the increased hysteresis losses may give rise to the enhanced heating capacity. Thirdly, it was reported that BMs have better crystallinity than synthetic MNPs [17], therefore, anisotropy of the nanoparticles (shape or magnetocrystalline) could be the other factor that influences the heating effect [10]. Of course, the exact mechanisms still need further exploration.

The unique features of nanoparticles will decide their behaviors in MFH. In the present work, we compared the difference in physicochemical properties of MNPs modified with amino silane and BMs. From the characterization analysis we can see that the magnetic properties of BMs and MNPs are different. Synthetic MNPs show superparamagnetic characteristics with zero hysteresis cycle, while BMs are ferromagnetic, existing hysteresis losses. It has been shown that the transition from superparamagnetic to ferromagnetic behavior occurs at a critical size of 25 nm for ultra fine magnetically ordered particles [18]. Thus, the different magnetic properties of BMs and MNPs may be largely due to their different size. We can also see that the saturation magnetization of MNPs (65.227 emu/g) is larger than that of BMs (34.140 emu/g), and this may be due to the presence of the nonmagnetic MMs of 3–5 nm thickness outside the BMs. It has been demonstrated that the MMs is not only critical for the control of crystal size and morphology, but also prevents the aggregation of extracted magnetosomes and thus stabilizes magnetosome suspensions [4]. So, the presence of biocompatible phospholipid membranes around BMs is one of its outstanding peculiarities compared with the artificial MNPs, with some functional groups on its surface as determined by FTIR.

Traditional therapeutic temperature range in hyperthermia against tumor is 42 °C–45 °C [1]. This approach can destroy tumors with minimal damage to healthy tissues. We found in the previous study that a higher heating dose above 45 °C could kill more cancerous cells, requiring less time of therapy and is able to stimulate the autologous immunity of the patients [19], thus the therapy effects are more satisfactory. Accordingly, the heating dosages we used in the present paper were all between 45 °C and 50 °C. In water-bath hyperthermia, we could see that compared with tumor cells MCF-7, normal cells L929 are less sensitive to heat, showing a relatively higher survival rate. In MFH in vivo, the heating dosage of 47 °C was chosen because the inhibitory rate of heat against tumor cells was high, and it is a relatively safe dosage. The inhibitory rate of ~80% was relatively lower than that of it in water-bath hyperthermia at 47 °C (62.2%), indicating the MFH protocol actually resulted in a slightly higher thermal dose compared

with hot water protocol, which is consistent with the previous studies [20]. Moreover, we found that compared with MNPs modified with amino silane, less BMs are needed to reach the therapeutic temperature, indicating the heating effect of BMs is better, which is favorable for the application in the future, because dosages of magnetic fluids could be minimized.

Biocompatibility of amino silane-modified MNPs and BMs was evaluated in the form of acute toxicity in mice. Different dosages of the two kinds of particles were utilized according to the preliminary test. We can see from the results that 50% of the mice died when administrated with 180 mg/kg MNPs, and only one mouse died in the highest dosing group of BMs (480 mg/kg), indicating the toxicity of BMs is weaker than that of MNPs modified with amino silane, although mice exposed to BMs were slightly listless during the first 4 days. LD_{50} is an important value for evaluation of biocompatibility, but we have not calculated the accurate LD_{50} because it would require more mice to die, and the aim of the present paper is only to compare the acute toxicity between MNPs and BMs. However, it can be supposed that the LD_{50} of MNPs is around 180 mg/kg, and the LD_{50} of MNPs will exceed 480 mg/kg. This prediction of LD_{50} of BMs is much higher than the previous study [21], we analyze that it may attribute to the following reasons: first, compared with mice, rats may be more susceptible to the particles, as sensitivity could be different between species. Secondly, different approaches of evaluating acute toxicity were utilized. In the previous study, ''up-and-down'' procedure was used, and only one rat was used per group for evaluating. In the present study, ten mice were used per group as mortality was required to compare the toxicity of MNPs and BMs, so the result may be more accurate. Anyhow, we could see that BMs may serve as a new potential material in MFH.

From the pathological sections, TEM images and organ coefficient analysis after mice were sacrificed, it could be seen that spleen and liver are the two major organs where nanoparticles distribute, and they are supposed to be digested by lysosomes. So the reticuloendothelial system, which is mainly comprised with macrophages of livers and spleens, is supposed to remove the particles from the bloodstream after intravenous administration, as detected in other studies [22]. In addition, decomposition of the two kinds of nanoparticles was analyzed in vitro, simulating the intracellular lysosomal conditions. We could see that BMs are more easily decomposed, with only remnants in 42 days time. That is a good phenomenon because it indicates that the biosynthetic BMs may be more easily eliminated by the organisms after they produce their effects, thereby exhibiting a lower toxicity.

As a pioneer study, the present work introduced the biomaterial BMs as energy-absorption agents to be used in MFH. It could be heated under AMF and induce cells to necrosis, the compatibility of BMs with tissues is high. In the future, in vivo studies aiming to evaluate the tumor ablating effects using BMs should be developed, and the toxicological profile of the nanoparticles and their in vivo fate after long-term survival in the body should be documented. Of course, since only amino silane-modified MNP was used in the study, the difference between BMs and MNPs modified with other methods deserves more research. Hopefully, this work helps introduce biologists and medical scientists to the tremendous potential of this young field and, in the process, inspires some to join the small but growing list of magnetosome researchers.

5. Conclusions

To sum up, by comparing with MNPs modified with amino silane, which is commonly used in biomedicine, BMs exhibit a better heating effect under AMF. Using MNPs and BMs of the same concentration, they could both enhance reduction in cell viability by hyperthermia, but current of lower intensity is needed by BMs to produce a similar inhibitory effect in the tumor cell. The acute toxicity evaluation in mice shows that the lethal dose BMs is much higher than MNPs, indicating the relatively high biocompatibility of BMs. Liver and spleen are the major organs where the two kinds of particles distribute.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.pnsc.2011.12.006.

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