Shape-controlled fabrication of magnetite silver hybrid nanoparticles with high performance magnetic hyperthermia

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Abstract
Superparamagnetic Fe3O4 nanoparticles (NPs)-based hyperthermia is a promising non-invasive approach for cancer therapy. However, the heat transfer efficiency of Fe3O4 NPs is relatively low, which hinders their practical clinical applications. Therefore, it is promising to improve the magnetic hyperthermia efficiency by exploring the higher performance magnetic NPs-based hybrid nanostructures. In the current study, it presents a straightforward in situ reduction method for the shape-controlled preparation of magnetite (Fe3O4) silver (Ag) hybrid NPs designed as magnetic hyperthermia heat mediators. The magnetite silver hybrid NPs with core-shell (Fe3O4@Ag) or heteromer (Fe3O4-Ag) structures exhibited a higher biocompatibility with SMMC-7721 cells and L02 cells than the individual Ag NPs. Importantly, in the magnetic hyperthermia, with the exposure to alternating current magnetic field, the Fe3O4@Ag and Fe3O4-Ag hybrid NPs indicated much better tumor suppression effect against SMMC-7721 cells than the individual Fe3O4 NPs in vitro and in vivo. These results demonstrate that the hybridisation of Fe3O4 and Ag NPs could greatly enhance the magnetic hyperthermia efficiency of Fe3O4 NPs. Therefore, the Fe3O4@Ag and Fe3O4-Ag hybrid NPs can be used to be as high performance magnetic hyperthermia mediators based on a simple and effective preparation approach.

1. Introduction

Colloidal hybrid nanoparticles (NPs), which include multiple components in a single nanosized object, are attractive for biomedical applications because novel important properties and multiple or enhanced functions can be generated due to the synergy of the individual component in the hybrid NPs [1–4]. Among the multitude of investigations to date, superparamagnetic NPs-based hybrid NPs have been considered as an important family of multifunctional or enhanced functional nanocomposites [5]. These types of NPs have shown great application potential in various biomedical fields such as drug delivery, magnetic resonance imaging and hyperthermia treatment [6]. Especially, based on the fact that magnetic NPs such as iron oxide NPs can generate heat when an alternating current magnetic field (ACMF) is applied to them [7,8], the magnetic hyperthermia is increasingly becoming one of the novel noninvasive approaches for cancer therapy. Cancer cells, more sensitive to temperature (≥40 °C) than healthy cells [9,10], can be selectively killed [11] by the magnetically induced heating, which can damage the biological integrity of the cell membrane and cytoskeleton [12]. However, the clinical trials has demonstrated that a considerably high concentration of iron oxide NPs [13,14] is needed for the efficient cancer thermal treatment because of the relatively poor heat transfer efficiency of current Fe3O4 NPs [15], which hinders their practical applications. In this regard, the...
development of high-performance hyperthermia mediators such as magnetic NPs attached therapeutic agents is of great importance to the practical applications of magnetic NPs-based hyperthermia [7,16,17].

Noble metallic silver (Ag) NPs have been recognized as a potential anti-cancer or antibacterial agent and extensively studied in the biomedical fields [18–20]. Recently, several recent studies have demonstrated that Ag NPs can enhance the efficiency of hyperthermia [13,21–24]. For example, Wang et al. [25] reported that Ag NPs improved the thermo-sensitivity of C6 cells. Liu et al. [13] studied the influence of a mixture of Fe3O4 and Ag NPs on magnetic hyperthermia, and found that the presence of Ag NPs enhanced the cancer cell death from magnetic heat both in vitro and in vivo. However, the heat generated by Fe3O4 NPs can only transmit very short distances and induce highly focused heating [15], and thus the separation of Fe3O4 and Ag NPs may limit the sensitivity of Ag NPs to Fe3O4 NPs-mediated hyperthermia.

It is promising to attain the lattice-mismatched heterojunctions by tailor-made chemical combining Fe3O4 and Ag NPs into a single unit; this would integrate the properties of the two NPs as well as enhance the functions between them at nanoscale level [26–29]. Various approaches have been reported for the preparation of Fe3O4-based magnetite silver hybrid NPs. However, little has been achieved with various morphologies, including centric or eccentric core/shells [30–32], phase-segregated core-satellites [33–35] and heteromer architectures [36,37]. However, the study on shape control of the magnetite silver hybrid NPs is still scarce [38–40].

In this study, we present a simple and effective in situ reduction strategy to prepare the magnetite silver hybrid NPs. It designed the pre-existing carboxyl-rich Fe3O4 NPs to carry inorganic Ag bonding junctions [2] in order to overcome the high interfacial energy barrier. Based on this design strategy, the shape of magnetite hybrid NPs can be tuned easily by adjusting the concentration of the intermediate product Fe3O4-COOAg NPs to produce core-shell Fe3O4@Ag or heteromer Fe3O4-Ag structures after in situ reduction. The cytotoxicity of the magnetite silver hybrid NPs is much lower than the individual Ag NPs. Compared to the individual Fe3O4 NPs, both Fe3O4@Ag and Fe3O4-Ag NPs exhibited greatly enhanced hyperthermia effects in vitro and in vivo. To the best of our knowledge, so far, this is the first case in which magnetite silver hybrid NPs were applied to in vitro and in vivo magnetic hyperthermia, thereby providing a novel and effective approach to improve the hyperthermia efficiency.

2. Materials and methods

2.1. Preparation of magnetite silver hybrid NPs

2.1.1. Synthesis of monodisperse hydrophobic Fe3O4-OA NPs

The Fe3O4-OA NPs (OA: oleic acid) were prepared by the classical thermal decomposition method [41,42]. In a typical chemical preparation, iron chloride (FeCl3·6H2O, 2.16 g) and sodium oleate (7.3 g) were dissolved in a mixture solvent consisting of ethanol (16 mL), pure water (12 mL) and hexane (28 mL). The mixture was stirred for 4 h at 70 °C. Then the upper organic layer containing the iron-oleate complex was washed three times with pure water in a separating funnel, and subsequently, hexane was evaporated off. The as-prepared iron-oleate complex was collected and dried overnight in a drying oven at 40 °C under vacuum. Then, the iron-oleate complex (3.4 g) and oleic acid (500 μL) were dissolved in the high boiling 1-octadecene solvent (20 mL). With the protection of nitrogen, the solution was programmed heated from the room temperature to 320 °C at 3.3 °C/min, and held isothermally for 30 min at 120 °C (open to air so as to remove trace water) and 320 °C, respectively. When the synthetic procedure was completed, the resulting mixture was cooled, and the Fe3O4-OA NPs were precipitated by ethanol (50 mL) and separated by centrifugation. The NPs were washed 3 times with 1:3 hexane and acetone, and collected by centrifugation, and finally dried under vacuum. The obtained Fe3O4-OA NPs were in a black solid form.

2.1.2. Synthesis of hydrophilic Fe3O4-PAA NPs

Fe3O4-PAA NPs (PAA: Poly(acrylic acid)) were prepared following a previously reported method [43]. In a typical process, under an inert nitrogen atmosphere, diethylene glycol (DGE, 35 mL) mixed with PAA (2 g) was heated to 120 °C with strongly stirring with the injection of the prepared Fe3O4-OA NPs toluene solution (100 mg/mL). Then the mixture was heated to 210 °C under reflux for 1 h. After the mixture was cooled down to room temperature, a brown–black magnetic powder was collected by the addition of dilute hydrochloric acid and magnetic separation. The precipitate of Fe3O4-PAA NPs was washed several times with pure water, and then well dispersed in pure water by ionizing the carboxyl groups with triethylamine to reserve. Fe3O4-PAA NPs were purified by ultrafiltration with an Ultra centrifugal filter unit (Millipore, 100,000 NMWL).

2.1.3. Synthesis of core-shell magnetite silver hybrid NPs (Fe3O4@Ag)

Aqueous solutions of Fe3O4-PAA NPs (1.5 mg/mL, 13.3 mL) and silver nitrate (AgNO3, 40 mg/mL, 10 mL) were mixed and shaken vigorously on a micro-oscillator for 20 min. A massive precipitate of Fe3O4-COOAg NPs was produced and collected by magnetic separation. The precipitate was ultrasonically redissolved in an aqueous solution of sodium citrate (Na3Cit, 10.6 mmol/L, 16 mL). After 12 h stirring and membrane filtration (220 nm), a saturated solution of Fe3O4-COOAg NPs was obtained. Then the saturated solution of Fe3O4-COOAg NPs (5.55 mg Ag/mL, 15 mL) was stirred and added with aqueous solution of freshly prepared sodium borohydride (NaBH4, 26 mmol/L, 100 μL) in every 10 s to a total volume of 1 mL, and stirring was continued for 30 min. The resulting mixture was purified by ultrafiltration to yield Fe3O4@Ag.

2.1.4. Synthesis of heteromer magnetite silver hybrid NPs (Fe3O4-Ag)

A saturated solution of Fe3O4-COOAg NPs (5.55 mg Ag/mL, 15 mL) was diluted with an aqueous solution of Na3Cit (10.6 mmol/L) to prepare an unsaturated solution of Fe3O4-COOAg NPs (4.37 mg Ag/mL). Then an aqueous solution of freshly prepared NaBH4 (26 mmol/L, 100 μL) was added in every 10 s to a total volume of 1 mL under stirring. The solution was stirred for 30 min, and purified by ultrafiltration to yield Fe3O4-Ag.

2.2. Experimental setup and measurements for magnetic hyperthermia

The magnetically induced heating efficiency of Fe3O4-PAA, Fe3O4@Ag and Fe3O4-Ag NPs was measured and recorded with a commercial system (illustrated in Fig. S1) with a moderate power frequency (390 KHz) heating machine equipped with an inductive copper coil of 3 turns, an inner diameter of 31 mm, an outer diameter of 41 mm (Shuangping SPG-06-II, China); and a fiber optic temperature sensor (UMIIJ universal multichannel, FISO Technologies Inc., Quebec, Canada) fitted with a pre-calibrated fiber optic temperature probe (FISO, model FOT-L-SD-C1-F1-M2-R1-ST). The prepared NPs at a concentration of 2 mg Fe/mL were placed inside the copper coil of the applied ACMF (390 KHz, 18 A). Specific absorption rate (SAR) was used to quantify the heating efficiency generated per unit gram of Fe per unit time [56], expressed as Equation (1):
where C is the specific heat capacity of the solution (here, the heat capacity of the solvent, \( C_{\text{water}} = 4.185 \, \text{J/g} \cdot \text{°C} \)), \( \frac{dT}{dt} \) is the initial slope of the temperature-time curve, \( m_s \) is the mass of the suspension, and \( m_m \) is the mass of the Fe content in the suspension.

2.3. Cell culture and animals

Human hepatoma cell line SMMC-7721 and human normal hepatocyte cell line L02 were purchased from KeyGen Biology Technology Company (Nanjing, China). SMMC-7721 cells and L02 cells were cultured in RPMI 1640 medium (KeyGen Biology, China) supplemented with 10% fetal bovine serum (FBS, Sijiqing, Hangzhou, China) and incubated at 37 °C in a 5% CO2 incubator (HERA cell 150, Thermo Electron Corporation).

BALB/c nude mice (female, aged 4 weeks) were purchased from the Comparative Medicine Center of Yangzhou University (Yangzhou, China). All animal studies were carried out according to the Guidelines for Animal Experimentation with the approval of the Animal Care Committee of Southeast University, Nanjing, China.

2.4. In vitro cytotoxicity test

The in vitro cytotoxicity of Fe3O4-PAA, Fe3O4@Ag and Fe3O4-Ag NPs in SMMC-7721 cells and L02 cells was assessed using an MTT assay. MTT (250 \( \mu \)L per well, and the cells were cultured for 4 h) was dissolved in dimethyl-sulfoxide (DMSO, 150 \( \mu \)L) containing formazan was transferred to a 96-well plate prior to the measurement of absorbance at 490 nm by a microplate reader. The calculation of relative cell inhibition was carried out as

\[
\text{Relative cell inhibition} = \frac{[\text{OD}_{\text{control}} - \text{OD}_{\text{background}}]}{\text{OD}_{\text{treated}} - \text{OD}_{\text{background}}} \times 100\%.
\]


d is the injection does of all of the Fe3O4-based NPs was normalized to be 15 mg Fe/Kg body weight. Particularly for hyperthermia groups of 6–8, the tumors were given to ACMF treatments at 2 h after each injection. In seven day follow-up experiment, the tumors in groups 2 and 6–8 were exposed to ACMF for 20 min, repeating 6 times (days 1–6) at 24 h intervals (in vivo therapeutic protocol diagram see Fig. 7a). Tumor-bearing mice were placed into the copper coil of the applied ACMF (390 KHz, 18 A) using a specially made polypropylene supporter so that tumors were positioned in the middle of the coil interior space possessing the highest field density of ACMF (Fig. S2). The temperature of the tumors was monitored with an infrared thermometer (FLUKE thermal imager, Ti32 model). In addition, the mice were weighed by electronic balance and their tumor growth were measured by vernier caliper on an alternate day. The tumor volumes were calculated as

\[
V = \frac{d^2 \times D}{2} \quad \text{(where } d \text{ and } D \text{ represent the shortest and longest diameter of the tumor in mm, respectively).}
\]

The relative tumor volume was calculated as

\[
\text{Relative tumor volume} = \left( 1 - \frac{V_{\text{treated, group}}}{V_{\text{control, group}}} \right) \times 100\%.
\]

2.5. In vitro magnetic hyperthermia experiment

SMMC-7721 cells (5 \( \times \) 10^4) were grown in sterile glass Petri dishes (specially made, 22 mm in diameter and 30 mm in height) in a 5% CO2 incubator at 37 °C for 24 h. After removing the medium, cells were incubated with 1 mL medium containing Fe3O4-PAA, Fe3O4@Ag and Fe3O4-Ag NPs at a final concentration of 15, 30 and 60 \( \mu \)g Fe/mL in duplicate, which was representative of two groups as follows: non-hyperthermia groups: Fe3O4-PAA, Fe3O4@Ag and Fe3O4-Ag NPs group; hyperthermia groups: Fe3O4-PAA plus ACMF, Fe3O4@Ag plus ACMF and Fe3O4-Ag NPs plus ACMF group. Non-hyperthermia groups were incubated for 12 h. Hyperthermia groups were incubated for 12 h, and then subjected to ACMF (390 KHz, 18 A) for 20 min. Cell inhibition was measured using an MTT assay. MTT (250 \( \mu \)L, 1 mg/mL) was added to each Petri dish and incubated with cells for 4 h. After removing the supernatant, DMSO (750 \( \mu \)L) per dish was added to dissolve the formazan. DMSO (150 \( \mu \)L) containing formazan was transferred to a 96-well plate prior to the measurement of absorbance at 490 nm by a microplate reader. The calculation of relative cell inhibition was carried out as

\[
\text{Relative cell inhibition} = \frac{[\text{OD}_{\text{control}} - \text{OD}_{\text{background}}]}{\text{OD}_{\text{treated}} - \text{OD}_{\text{background}}} \times 100\%.
\]

2.6. In vivo magnetic hyperthermia experiment

The tumor models were established by subcutaneous injection of SMMC-7721 cells (1 \( \times \) 10^5 cells/mouse) into the right flanks of nude mice. When the volume of the tumors reached about 300 mm^3, the mice were randomly divided into eight groups (n = 6/group): (1) saline group (negative control), (2) saline plus ACMF group, (3) Fe3O4-PAA NPs group, (4) Fe3O4@Ag NPs group, (5) Fe3O4@Ag NPs group, (6) Fe3O4-PAA NPs plus ACMF group, (7) Fe3O4-Ag NPs plus ACMF group, (8) Fe3O4@Ag NPs plus ACMF group. Following a multipoint injection strategy, moving clockwise at the 3, 6, 9 and 12 o’clock points, the mice were intratumorally injected with aqueous solution of Fe3O4-PAA, Fe3O4-Ag, Fe3O4@Ag NPs and saline, respectively, on the 1st and 4th day. The single injection does of all of the Fe3O4-based NPs was normalized to be 15 mg Fe/kg body weight.

2.7. Statistical analysis

Values were expressed as mean ± SDs. The data were analyzed using the unpaired t-test, and a P value of <0.05 was considered as statistically significant difference.

3. Results and discussion

3.1. Synthesis and characterization of magnetite silver hybrid NPs

The synthesis of magnetite silver hybrid NPs is depicted schematically in Scheme 1. First, the excellent superparamagnetic and monodispersed Fe3O4-OA NPs core were prepared by the classical thermal decomposition of iron oleate in 1-octadecene. Short-chain PAA with abundant free carboxyl groups was used to replace of OA on Fe3O4 NPs surface to achieve carboxyl-rich Fe3O4-OA NPs via the ligand exchange reaction. The hydrophilic carboxyl-rich Fe3O4-OA NPs then provided the abundant anchor sites of Ag source on
their surfaces. Then, because of a strong affinity of ionized carboxyl group to Ag⁺ ion \[44\], the abundant Ag⁺ ions were bonded to the surface of Fe₃O₄ NPs by excess of AgNO₃ added resulting in the Fe₃O₄-COOAg NPs precipitate \[45\]. This precipitate was redissolved in different amounts of Na₃Cit solution to yield different concentration Fe₃O₄-COOAg NPs solutions. During this process, it was found that the concentration of Ag in these solutions may be a key factor for the shape control of magnetite silver hybrid NPs. After in situ reduction with NaBH₄, core-shell Fe₃O₄@Ag hybrid NPs were obtained at a concentration of 5.55 mg Ag/mL (the saturated concentration of the Fe₃O₄-COOAg NPs solution), and heteromer Fe₃O₄-Ag hybrid NPs were obtained at a concentration of 4.35 mg Ag/mL.

It is well-recognized that the high density of carboxyl groups on magnetic NPs surface \[43,46\] can help create the formation of the core-shell \[47\] or core-satellite \[27,48\] Fe₃O₄@Ag hybrid structure. Encouraged by the existing precipitation-dissolution behavior of the precipitate of Fe₃O₄-COOAg NPs (Fig. S3), the possible mechanism of magnetite silver hybrid NPs shape control may be associated with the ionization equilibrium of the Fe₃O₄-COOAg NPs solution shown in Equation (2) \[49\].

\[
\text{Fe}_3\text{O}_4 - \text{COO}^-\text{Ag}^{+\text{(s)}} \rightleftharpoons \text{Fe}_3\text{O}_4 - \text{COO}^-\text{Ag}^{+\text{(aq)}} \rightleftharpoons \text{Fe}_3\text{O}_4 - \text{COO}^-\text{(aq)} + \text{Ag}^{+\text{(aq)}}
\]

(2)

According to the Le Chatelier’s principle, the equilibrium moves to the right when the solution is diluted, and moves to the left when the solution is concentrated. The smaller the concentration of Fe₃O₄-COOAg NPs (aq), the greater the dissociation degree of [-COO⁻] and [Ag⁺] (the proving experiment see S1.4). We hypothesized that controlling the dissociation degree of [-COO⁻] and [Ag⁺] could rebuild site-differential surface distributions of Ag source on the surface of Fe₃O₄-COOAg NPs (aq). When in a saturated Fe₃O₄-COOAg NPs solution (5.55 mg Ag/mL), the minimized dissociation of [-COO⁻] and [Ag⁺] was kept; and most of Ag⁺ ions were bounded on the carboxyl groups of the Fe₃O₄-COOAg NPs (aq) surface. Thus,
the core-shell Fe$_3$O$_4$@Ag hybrid NPs were formed after in situ reduction (Scheme 1). When the Fe$_3$O$_4$@COOAg NPs solution was diluted to 4.35 mg/mL, the dissociation degree of [-COO\textsuperscript{-}] and [Ag\textsuperscript{+}] was increased. Under this condition, only partial Ag\textsuperscript{+} ions were still bounded on the carboxyl groups of the Fe$_3$O$_4$-COOAg NPs (aq) surface to generate the heteromer Fe$_3$O$_4$-Ag hybrid NPs after in situ reduction (Scheme 1).

The morphology of magnetite silver hybrid NPs was characterized by transmission electron microscope (TEM). Fe$_3$O$_4$@Ag hybrid NPs (Fig. 1b) are spherical and uniform with a diameter of 10.41 ± 0.95 nm, which is similar to 10.1 ± 0.98 nm of Fe$_3$O$_4$-PAA NPs (Fig. 1a, the size distribution histograms see Fig. S4), indicating that the Ag shell is very thin. The High-resolution TEM (HRTEM) image provides detailed structural information of the core-shell structure (Fig. 1b). Both the lattice fringe spacing of ~0.253 nm and ~0.237 nm, corresponding to the (311) planes of the cubic spinel structured Fe$_3$O$_4$ (JCPDS card no. 19-0629) and (111) planes in the face-centered cubic Ag (JCPDS card no. 04-0783) respectively, can be observed in a single Fe$_3$O$_4$@Ag hybrid NP (Fig. 1d), confirming the coexistence of Ag and Fe$_3$O$_4$ crystals in a single hybrid NP. Ag generally appears darker than Fe$_3$O$_4$ in TEM images because it possesses a high electron density compared to Fe$_3$O$_4$ [50], and thus it is not easy to confirm the core-shell structure of Fe$_3$O$_4$@Ag NPs by TEM images. To further confirm the core-shell structure of Fe$_3$O$_4$@Ag hybrid NPs, a single Fe$_3$O$_4$@Ag NP was further analyzed by high-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM). As shown in Fig. 1f, the NP was much brighter than the Fe$_3$O$_4$ portions of the heteromers shown in Fig. 1g. Ag appears brighter than Fe$_3$O$_4$ because of the higher intensity of scattered electrons in the HAADF-STEM image [50], which demonstrates the existence of an Ag shell in the Fe$_3$O$_4$@Ag NP. The energy dispersive X-ray spectroscopy (EDS) analysis of a single Fe$_3$O$_4$@Ag NP further confirmed the coexistence of iron and Ag elements in the hybrid NP (Fig. 1h).

The morphology of the heteromer Fe$_3$O$_4$-Ag hybrid NPs was also characterized by TEM, HRTEM, HAADF-STEM and EDS. As shown in the TEM image (Fig. 1c), the structure of a single heteromer NP was composed of one NP of ~10 nm bearing 1–3 NPs of 2–5 nm. From the HRTEM image of the heteromer NP (Fig. 1e), it can be seen that the lattice fringe spacings of the NP of ~10 nm were ~0.253 nm and ~0.297 nm, corresponding to the (311) and (220) planes of Fe$_3$O$_4$ (JCPDS card no. 19-0629). The smaller NPs attached to the Fe$_3$O$_4$ had a lattice fringe spacing of ~0.237 nm, corresponding to the (111) plane of fcc Ag (JCPDS card no. 04-0783). As expected, in the HAADF-STEM image (Fig. 1g), the Ag portions of the heteromer NPs were much brighter than the Fe$_3$O$_4$ portions. In addition, EDS analysis of a single heteromer NP confirmed the coexistence of iron and Ag elements in the heteromer (Fig. 1i). These results demonstrate that the two types of magnetite silver hybrid NPs are core-shell and heteromer structures, respectively. The constituents of

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**Fig. 1.** TEM images of Fe$_3$O$_4$-PAA (a), Fe$_3$O$_4$@Ag (b) and Fe$_3$O$_4$-Ag (c) NPs; HRTEM images of Fe$_3$O$_4$@Ag (d) and Fe$_3$O$_4$-Ag (e) NPs; HAADF-STEM images of Fe$_3$O$_4$@Ag (f) and Fe$_3$O$_4$-Ag (g) NPs; EDS patterns of Fe$_3$O$_4$@Ag (h) and Fe$_3$O$_4$-Ag (i) NPs (the inserts are the scanning areas corresponding to the EDS spectra).
the two types of hybrid NPs were further qualified by ICP-MS. The molar ratio of Fe to Ag in Fe3O4@Ag is 1:0.22 for Fe3O4@Ag hybrid NPs, and Fe to Ag in Fe3O4-Ag is 1:0.17 for Fe3O4-Ag hybrid NPs.

The crystal structures of magnetite silver hybrid NPs were characterized by powder X-ray diffraction (XRD). As shown in Fig. 2a, the XRD spectra of Fe3O4@Ag and Fe3O4-Ag hybrid NPs were very similar. In both spectra, the reflections of 30.22°, 35.46°, 43.20°, 57.28° and 62.74° (marked with asterisks) can be attributed to the (220), (311), (400), (511) and (440) planes of cubic-phase Fe3O4 (JCPDS Card, No. 19-629, see Fig. S5), and the reflections of 38.14° and 44.42° (marked with rhombi) can be attributed to the (111) and (200) planes of the fcc Ag (JCPDS Card, No. 4-783, see Fig. S6). This demonstrates that both Fe3O4@Ag and Fe3O4-Ag NPs are composed of crystalline Fe3O4 and Ag.

The optical properties of magnetite silver hybrid NPs were characterized using UV–vis absorption spectra (Fig. 2b). Both Fe3O4@Ag and Fe3O4-Ag hybrid NPs showed intense peaks at 407 nm and 416 nm, respectively, which can be attributed to the surface plasmon resonance (SPR) effect of nano Ag. The SPR peaks of Fe3O4@Ag and Fe3O4-Ag NPs were red-shifted to different degrees compared to that of Ag NPs, which is due to the local dielectric effect according to classical Mie theory [51]. Similar red-shifts have also been observed previously [39,51].

3.2. Magnetically induced heating characteristics of magnetite silver hybrid NPs in ACMF

VSM was used to evaluate the magnetic properties of these two types of magnetite silver hybrid NPs for future magnetic hyperthermia. Results shown in Fig. 3a indicate that both Fe3O4@Ag and Fe3O4-Ag hybrid NPs exhibited superparamagnetic properties similar to Fe3O4-PAA NPs. The saturation magnetizations (Ms) of Fe3O4-PAA, Fe3O4-Ag and Fe3O4@Ag NPs were 82.4, 78.1 and 75.1 emu/g Fe, respectively. Although the saturation magnetization of both Fe3O4-Ag and Fe3O4@Ag NPs has a little bit decrease, the presence of Ag in the hybrid NPs had no significant effect on the magnetic properties of Fe3O4.

To further investigate the magnetically induced heating properties of magnetite silver hybrid NPs, the heating efficiency of Fe3O4-PAA, Fe3O4-Ag and Fe3O4@Ag NPs were studied. SAR was used to quantify the heating efficiency generated per unit gram of Fe per unit time. Three samples, 1 mL each of Fe3O4-PAA, Fe3O4-Ag and Fe3O4@Ag NPs aqueous solutions at a concentration of 2 mg/mL Fe, were placed inside an ACMF with a moderate radio frequency of

![Fig. 2.](image)

(a) X-ray diffraction patterns of Fe3O4@Ag and Fe3O4-Ag hybrid NPs. (b) UV–vis absorption spectra of the aqueous solutions of Fe3O4-PAA, Fe3O4@Ag, Fe3O4-Ag and Ag NPs.

![Fig. 3.](image)

(a) Hysteresis loops of Fe3O4-PAA, Fe3O4-Ag and Fe3O4@Ag NPs. (b) Temperature-time curves of aqueous solutions of Fe3O4-PAA, Fe3O4-Ag, Fe3O4@Ag NPs and pure water with Fe concentration of 2 mg/mL as a function of the ACMF exposure time.
390 kHz and a current of 18 A. As shown in Fig. 3b, the temperatures of all samples rose approximately 12 °C after 600 s ACMF treatment; and the SAR values were 87, 83 and 76 W/g for Fe₃O₄-PAA, Fe₃O₄-Ag and Fe₃O₄@Ag NPs, respectively. The SAR result indicates that the existence of Ag in the two latter hybrid NPs had no significant influence on their magnetically induced heating. Moreover, the pure water exposed to the applied ACMF showed only a negligible temperature increase, indicating that the temperature rise of the samples can be attributed to the magnetically induced heating effect of the NPs.

3.3. Evaluation of in vitro cytotoxicity

To use magnetite silver hybrid NPs in living systems, it is important to assess their stability and biocompatibility. Dynamic light scattering (DLS, Zeta Plus Particle Size Analyzer, Brookhaven, US) was used to determine the hydrodynamic diameters of Fe₃O₄-PAA, Fe₃O₄-Ag and Fe₃O₄@Ag NPs in aqueous solution, biological medium and biological medium suffering from ACMF, which exhibited the high stability level (Table S1). The cytotoxicity of each type of NPs with various Fe concentrations was evaluated against SMMC-7721 cells and L02 cells using a MTT assay. As shown in Fig. 4a and 4d, after treatment with Fe₃O₄@Ag, Fe₃O₄-Ag or Fe₃O₄-PAA NPs for 24 h, the cell viability was above 90% at Fe concentrations up to 60 μg/mL, indicating that magnetite silver hybrid NPs have no obvious cytotoxicity at the tested concentrations. As comparison, we also tested the cytotoxicity of the individual Ag NPs and found that its toxicity was evident at Ag concentrations greater than 10.35 μg/mL (Fig. 4b, 4c, 4e and 4f). In contrast, Fe₃O₄-Ag and Fe₃O₄@Ag hybrid NPs showed no significant cytotoxicity at Ag concentrations up to 24.83 μg/mL. This demonstrates that the incorporation of Ag into magnetite silver hybrid NPs reduces the toxicity of Ag.

3.4. In vitro magnetic hyperthermia for cancer cells

Next, the in vitro use of the prepared magnetite silver hybrid NPs for magnetic hyperthermia in SMMC-7721 cells was evaluated. To maximize the heat generation of the magnetic NPs and minimize the background heating effect of the eddy current induced by ACMF, the applied ACMF was set at a moderate 390 KHz frequency and a current of 18 A (Optimization shown in Fig. S7) [52,53]. Cell inhibition was examined by MTT assay after 12 h incubation with a series of doses of Fe₃O₄@Ag, Fe₃O₄-Ag and Fe₃O₄-PAA NPs, followed with or without a 20-min exposure to ACMF. Fig. 5 shows that the cell inhibition rates were 51%, 75% and 83% for Fe₃O₄@Ag NPs plus ACMF treatment and 46%, 72% and 75% for Fe₃O₄-Ag NPs plus ACMF treatment at non-cytotoxic concentrations of 15, 30 and 60 μg Fe/mL, respectively, which were significantly higher than the rates for Fe₃O₄-PAA NPs plus ACMF treatment at the corresponding concentrations. It’s believed that both Fe₃O₄@Ag NPs and Fe₃O₄-Ag NPs have higher inhibition ability for SMMC-7721 cells than Fe₃O₄-PAA NPs; magnetic hyperthermia efficiency was greatly enhanced by using Fe₃O₄@Ag and Fe₃O₄-Ag NPs compared to Fe₃O₄-PAA NPs.

The results demonstrate that the efficiency of in vitro magnetic hyperthermia can be greatly enhanced in the presence of Ag incorporated into magnetite silver hybrid NPs. The higher inhibition may come from silver ions released from the surface of Fe₃O₄@Ag and Fe₃O₄-Ag NPs. Silver ions released from the particles surface are considered to be the principal mechanism of Ag NPs toxicity [54]. On the one hand, silver ions readily bind to sulfur- and
phosphorus-containing biomolecules such as proteins and DNA, thereby potentially causing cell damage [55]. On the other hand, silver ions could also interfere with mitochondrial activity and induce apoptosis [20]. Several investigations have reported that Ag\(^{+}\) ions are released from Ag NPs in aerobic aqueous environments [56]. The dissolution of Ag NPs to silver ions is an oxidation process. Upon the internalization of Ag NPs by cells, the rate of intracellular oxidation of Ag NPs is higher compared with that in water due to higher intracellular oxygen level [57,58]. Also, Ag NPs could be further dissolved to silver ions in the lysosomes due to the lower pH environment [59]. TEM images in Fig. 6 indicate the cellular uptake and localization of magnetite silver hybrid NPs in the absence and presence of ACMF. The results showed that all the NPs could indeed be internalized and localized in the cytoplasm and lysosomes with and without ACMF treatment. The localization in acid lysosome would enhance the release of silver ion from the hybrid NPs. In addition, Heat, including photothermal [22,23,60] and magnetically induced heating [13,25], could trigger the release of Ag\(^{+}\) ions. We further compared \textit{in vitro} ionic silver released from the two hybrid NPs in aqueous solution with and without exposure to ACMF. A negligible (0.04%) increase in Ag\(^{+}\) ion release from Ag NPs was observed with ACMF treatment compared to without ACMF. However, 1.2% and 3.1% increases in Ag\(^{+}\) ion release from Fe\(_3\)O\(_4\)@Ag and Fe\(_3\)O\(_4\)-Ag NPs were observed with ACMF compared to without ACMF, indicating a boosting of silver ions release. Taken together, magnetic hyperthermia efficiency was significantly enhanced using Fe\(_3\)O\(_4\)@Ag and Fe\(_3\)O\(_4\)-Ag hybrid NPs compared to Fe\(_3\)O\(_4\)-PAA NPs at a series of non-cytotoxic concentrations, suggesting a possibly synergistic effect of magnetically induced heating and heat-assisted silver ions release. The inhibition rate of the Fe\(_3\)O\(_4\)@Ag was slightly higher than that of the Fe\(_3\)O\(_4\)-Ag, which may be due to the comparatively high Ag content in the core-shell structure.

3.5. \textit{In vivo} tumor magnetic hyperthermia for tumors

The \textit{in vivo} magnetic hyperthermia performance of the magnetite silver hybrid NPs was further investigated to assess their application potentials in cancer treatment. In the 7-day experiment, the two injections with saline, Fe\(_3\)O\(_4\)-PAA, Fe\(_3\)O\(_4\)-Ag and Fe\(_3\)O\(_4\)@Ag NPs aqueous solution (single dose 15 mg Fe/kg body weight) were administrated to saline group 1 (negative control); saline plus ACMF group 2; non-hyperthermia groups of Fe\(_3\)O\(_4\)-PAA NPs group 3, Fe\(_3\)O\(_4\)-Ag NPs group 4, Fe\(_3\)O\(_4\)@Ag NPs group 5; hyperthermia groups of Fe\(_3\)O\(_4\)-PAA NPs plus ACMF group 6, Fe\(_3\)O\(_4\)-Ag NPs plus ACMF group 7, Fe\(_3\)O\(_4\)@Ag NPs plus ACMF group 8, respectively, on the 1st and 4th day. And six ACMF treatments (390 KHz, 18 A, 20 min) were administrated to groups 2 and 6 at 24 h intervals on days 1–6. The intratumoral distribution of injected Fe\(_3\)O\(_4\)-based NPs were monitored by T2 and T2*-weighted MRI. The clear MR signal attenuation demonstrated that the injected NPs diffused to the whole tumor regions at 2 h post injections (Fig. S8).
The tumor temperature of the hyperthermia groups 6–8 was directly detected by the infrared imaging (FLUKE thermal imager, Ti32 model) after ACMF treatment. The thermal images displayed that the average temperatures of tumor surface could reached 40–43 °C (Table S2, a representative thermal image shown in Fig. S9). Since the temperature deviation between tumor interior and surface is usually ±2 °C [61], the temperature in our experiment is in the range of valid hyperthermia temperature, which were able to induce the apoptosis of tumor cells and inflict no damage to normal tissue [12]. After 7-day experiment, the evolution of tumor growth is presented in Fig. 7b for all the treated groups 1–8. The tumor volume inhibition rate is 65% for group 7 and 67% for group 8 respectively, which is significantly higher than 47% of group 6, 14% of group 5, 10% of group 4, 5% of group 3 and 3% of group 2. Even for comparison of non-hyperthermia groups 4–5 and group 1, Fe3O4-Ag and Fe3O4@Ag treatments exhibited significant inhibitions (P < 0.05) of tumor growth, which suggested that Fe3O4-Ag and Fe3O4@Ag hybrid NPs themselves have a certain inhibition effect due to the cytotoxicity derived from the presence of Ag in magnetite silver hybrid NPs; even for comparison of hyperthermia groups 6–8 and non-hyperthermia groups 3–5 treated with corresponding NPs, ACMF treatments exhibited extremely significantly higher inhibitions (P < 0.0001) of tumor growth, which suggested that magnetically induced heating can greatly enhance tumor growth inhibition effect. It is further noticed that there was no significant difference of tumor growth inhibition in the non-hyperthermia groups 4–5 compared to group 3, but an extremely significant difference (P < 0.001) in the hyperthermia groups 7–8 compared to group 6 under the condition of no obvious hyperthermia temperature difference. Considering statistical difference degree, it is believed that the combination of magnetite silver hybrid NPs and ACMF treatments can take a synergistically enhanced inhibition effect consistent with the results of in vitro magnetic hyperthermia. The tumor inhibition effects can also be observed intuitively from the typical tumor photographs recorded from each treated group during treatments (Fig. S10). In addition, there is no noticeable mortality and body weight discrepancies (Fig. 7c) between non-hyperthermia groups and hyperthermia groups compared with the saline group, indicating a well-tolerated dose level of the magnetite silver hybrid NPs.

To further assess the tumor therapy efficiency, all mice were sacrificed after treatments on the 7th day for H&E and TUNEL staining analysis (Fig. 8). Histopathology indicated that the combination of magnetite silver hybrid NPs and ACMF treatments induced massive complete necrosis and fibrosis symptoms of tumor tissues in hyperthermia groups 7–8, which were far more serious than that in non-hyperthermia groups 4–5 and hyperthermia group 6. This can further proof the excellent magnetic hyperthermia efficiency of magnetite silver hybrid NPs on the destruction of tumor cells. No obvious histopathological abnormalities or lesions were observed in the main organs (heart, liver, spleen and kidney) in all the groups, implying either the three NPs or magnetically induced heating to cancer therapy with low acute toxicity or little risk. Very small amounts accumulation of Fe3O4-PAA, Fe3O4-Ag and Fe3O4@Ag NPs were found in the liver and spleen using Prussian blue and nuclear fast red double staining (Fig. S11) in the corresponding treated groups. TUNEL assay was performed to evaluate whether tumor growth inhibition by treatment materials and magnetically induced heating was related to cell apoptosis. It was assuredly observed that the tumors treated with combination of magnetite silver hybrid NPs and ACMF treatments exhibited the extensive regions and high populations of apoptotic cells (brown cell) in hyperthermia groups 7 and 8. According to statistics, either the apoptosis rates 61.29± 14.38% for
group 7 or 63.26 ± 16.88% for group 8 were significantly higher than the rates 41.29 ± 10.3% for group 6, 15.69 ± 8.85% for group 5, 12.21 ± 5.2% for group 4, 7.91 ± 2.23% for group 3, 4.25 ± 1.98% for group 2, 2.17 ± 1.98% for group 1 (Table S3). The high apoptosis level in hyperthermia groups 7–8 may explain the greatly reduction in tumor size in these two groups.

4. Conclusion

In conclusion, an in situ reduction strategy to fabricate the lattice-mismatched Fe3O4 and Ag into a single nano-unit was developed. Using this method, the morphology of the magnetite silver hybrid NPs can be easily tuned to a core-shell (Fe3O4@Ag) or heteromer (Fe3O4-Ag) structure, which proved to a higher biocompatibility with SMMC-7721 cells and L02 cells than the individual Ag NPs. Both Fe3O4-Ag and Fe3O4@Ag hybrid NPs exhibited the significantly enhanced in vitro and in vivo tumor therapeutic effect compared to the individual Fe3O4 NPs, which may be attributed to the synergistic effect of magnetically induced heating and heat-assisted silver ions release. Therefore, the prepared magnetite silver hybrid NPs provide the potential better candidates for tumor magnetic hyperthermia treatment.

Conflict of interest

These authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2017.01.043.

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