Eumelanin-Fe$_3$O$_4$ hybrid Nanoparticles for Enhanced MR/PA Imaging-assisted Local Photothermolysis

Junqing Wang, F$^{a}$ Heng Liu,$^{a, b}$ Yu Liu,$^{a, c}$ Chengchao Chu,$^{a}$ Youyuan Yang,$^{b}$ Yun Zeng,$^d$ Weiguo Zhang,$^e$ and Gang Liu$^{a, d, f}$

In this work, we report a biodegradable eumelanin-Fe$_3$O$_4$ hybrid nanoparticles (euMel-Fe$_3$O$_4$ NPs) for multiple imaging-assisted local photothermolysis. The euMel-Fe$_3$O$_4$ NPs are rationally prepared via facile one-step co-precipitation method in aqueous phase in the presence of eumelanin. The obtained NPs exhibited high stability and aqueous dispersity. euMel-Fe$_3$O$_4$ NPs could serve for $T_2$-weighted magnetic resonance (MR) and photoacoustic (PA) imaging contrast enhancement prior to the treatment, due to their high $r_2$ relaxivity that was determined to be 245.88 mM$^{-1}$ s$^{-1}$ (a value higher than other commercially available iron oxide NPs) and good PA signal intensity. Besides, with effective NIR absorbance, the euMel-Fe$_3$O$_4$ NPs promoted an effective photothermolysis treatment in an experimental mouse model of cancer. Cytotoxicity and in vivo histological examination demonstrated no appreciable toxicity. Our work suggests that euMel-Fe$_3$O$_4$ NPs could be proposed as a potential class of theranostic nanoplatform with multiple imaging modalities for cancer therapy.

1. Introduction

Nanomaterial-assisted near-infrared (NIR) photothermolysis or thermal ablation therapy is being extensively studied as a more effective and less invasive alternative to surgical resection of solid tumors.\textsuperscript{1-6} Because the NIR light has deep tissue penetration (vary from few millimeter to 3 cm depth, dependent on the power of NIR light) with minimal damage to healthy tissues,\textsuperscript{7-10} Up to now, far too little attention has been paid to photothermolysis treatment by intratumoral interstitial approach using photothermal conversion agents (PTCAs). This single-dose and one-time treatment can not only afford high regional therapeutic concentrations while bypass BBB and systemic exposure, but can also avoid several limitations that had been encountered in the local chemo- and gene-therapeutic delivery. For example, multiple times of refilling, high toxic concentration, and catheter placement.\textsuperscript{11}

Although the most current PTCAs has demonstrated remarkable therapeutic efficacy, their safety profile has not yet been met with the criteria of clinical translation, due to rising concerns on non-degradability, poor metabolism, and toxicity.\textsuperscript{12-15} In addition, current biodegradable PTCAs lack the ability of clinical relevant diagnosis and monitoring for relatively deep tissues during treatment in real-time,\textsuperscript{16, 17} which inevitably limits their practical applications. Therefore, development of biodegradable PTA nanosystems (such as biomimetic organic and degradable inorganic materials) with transformative imaging capacity is urgently demanded.\textsuperscript{16, 18}

Magnetic iron oxide nanoparticles (IONPs) have been extensively explored for many years and have already achieved board biomedical and clinical applications ranging from disease diagnostics (e.g., nano-bio-sensing, MR contrast imaging, and magnetic particle imaging) to nanotherapeutics (e.g., controlled drug/gene delivery and magnetic hyperthermia).\textsuperscript{19-24} Intracellular degradation studies have found that IONPs undergo endosome-facilitated biodesintegration within a month after cellular internalization.\textsuperscript{25, 26}

Endogenous chromophores, typically melanin (a variety of biopolymers that are found in hair, skin, and eyes; biosynthesized via melanogenesis pathway), has been recognized as a promising photothermal therapeutic agent (PTA) due to the high photothermal conversion efficiency in NIR region, and the excellent biocompatibility and biodegradability in its nanoparticle form.\textsuperscript{24, 27-30} Melanin can be generally categorized into eumelanin, pheomelanin, and neuromelanin. The most common type is eumelanin, which consists of cross-linked 5,6-dihydroxyindole (DHI) and 5,6-
dihydroxyindole-2-carboxylic acid (DHICA) units in variable patterns. To date, several melanin-based PTAs have been reported with specific strategies for solid cancer therapy.\textsuperscript{16, 24, 27, 31, 32} In 2012, Lu et al. reported dopamine–melanin colloidal nanospheres (CNSs) produced by self-polymerization of dopamine in solvent mixtures. The resultant Dpa-melanin CNSs showed 40% of photothermal conversion efficiency.\textsuperscript{27} It’s in vivo study found that 4T1 and HeLa xenograft tumors could be photothermally ablated by readily heating up the tumor tissues above 50 °C within 5 min, after 808 nm laser (2 W cm\textsuperscript{-2}) irradiation.\textsuperscript{27} Studies performed by Chu et al. have demonstrated that liposome encapsulated with natural black sesame melanin (BSM) nanoparticles can passively target healthy and tumor-bearing mice SLNs (sentinel lymph nodes).\textsuperscript{24} This can be applied on intraoperative SLN mapping based on the black color staining effects of the nanoparticles.\textsuperscript{24} Recently, Jiang et al. developed an erythrocyte membrane-coated natural melanin nanoparticles for PA imaging-guided photothermolysis on A549 tumor-bearing mice. Such natural melanin nanoparticles demonstrated approximately 10% higher photothermal conversion efficiency than the melanin-like polydopamine nanoparticles.\textsuperscript{31} In addition, the erythrocyte coating endows immune evasion capability and enhances passive accumulation in tumor.\textsuperscript{31}

Inspired by the strong NIR absorbance, intrinsic Photoacoustic property,\textsuperscript{13, 34} and high iron-chelating capability of melanin,\textsuperscript{35, 36} we hereby present multifunctional eumelanin-Fe\textsubscript{3}O\textsubscript{4} hybrid nanoparticles (euMel-Fe\textsubscript{3}O\textsubscript{4} NPs) for magnetic resonance (MR)/Photoacoustic (PA) imaging-guided photothermal therapy of glioblastoma, through interstitial delivery in U-87 MG xenograft model. The euMel-Fe\textsubscript{3}O\textsubscript{4} NPs with 10–12 nm in size are rationally prepared via a facile one-step co-precipitation method in aqueous phase. The appearance of eumelanin on the surface endows euMel-Fe\textsubscript{3}O\textsubscript{4} NPs with desired dispersibility in water, and the injected NPs can rapidly induce the tumor hyperthermia up to 51.3 °C within 8 minutes upon NIR irradiation. Thus, promotes an effective photothermal treatment, followed by subsequent tumor cell apoptosis and necrosis. The developed euMel-Fe\textsubscript{3}O\textsubscript{4} NPs is also act as MR and PA contrast agents to evaluate the intratumoral distribution and the in vivo photothermalysis efficacy after interstitial delivery.

2. Materials and methods

2.1 Materials
Iron (III) chloride (FeCl\textsubscript{3}·6H\textsubscript{2}O), iron (II) chloride (FeCl\textsubscript{2}·4H\textsubscript{2}O), sodium hydroxide (NaOH), ammonium hydroxide (NH\textsubscript{4}OH), nitric acid (HNO\textsubscript{3}), hydroxyamine hydrochloride, o-phenanthroline, sodium acetate anhydrous (NaOAc) were first dissolved in 5 mL purified water. Purified water was produced by a Millipore water purification system. The chemical functionalities of euMel-Fe\textsubscript{3}O\textsubscript{4} NPs was characterized by XPS. Iron and eumelanin was determined by o-phenanthroline method ($O_2$) and absorption spectrum, respectively. Pristine Fe\textsubscript{3}O\textsubscript{4} NPs were prepared under similar condition without the presence of melanin.

2.2 Synthesis of euMel-Fe\textsubscript{3}O\textsubscript{4} NPs

EuMel-Fe\textsubscript{3}O\textsubscript{4} NPs were prepared by a modified co-precipitation method.\textsuperscript{37} In a typical synthesis, FeCl\textsubscript{3}·4H\textsubscript{2}O (100 µmol, 19.881 mg) and FeCl\textsubscript{2}·6H\textsubscript{2}O (200 µmol, 54.058 mg) were first dissolved in 5 mL of 1M HCl solution and heated to 90 °C in nitrogen atmosphere. Then, 0.5 mL NH\textsubscript{2}OH (28 vol.%) was added rapidly under vigorous stirring, followed by injecting 5 mL of eumelanin solution (1 mg/mL, pH = 9.0) 20 s later at a constant rate in 1 min. After that, the mixture was vigorously stirred at 90 °C for 30 min and cooled to room temperature. The product was collected by magnetic decantation, washed with deionized water several times until the pH value of the solution reached neutral. Finally, the obtained euMel-Fe\textsubscript{3}O\textsubscript{4} NPs was dispersed in 7.5 mL of deionized water, and stored at room temperature for further use. The concentration of iron and eumelanin was determined by o-phenanthroline method and absorption spectrum, respectively. Pristine Fe\textsubscript{3}O\textsubscript{4} NPs were prepared under similar condition without the presence of melanin.

2.3 Characterization

The morphology of euMel-Fe\textsubscript{3}O\textsubscript{4} NPs was characterized by TEM. The hydrodynamic diameter and zeta potential were measured by DLS (Zetasizer Nano ZS90, Malvern Instruments, UK). The UV-Vis spectra were recorded by using a UV-Vis spectrometer (Cary60, Agilent Technologies, USA). The chemical functionalities of euMel-Fe\textsubscript{3}O\textsubscript{4} NPs were characterized by FT-IR spectra. Magnetic property measurement was carried out with a vibrating sample magnetometer (VSM) on a Model 6000 physical property measurement system (Quantum, USA) at 300 K. X-ray diffraction (XRD) measurements were recorded to determine the composition of euMel-Fe\textsubscript{3}O\textsubscript{4} NPs. The X-ray photoelectron spectroscopy (XPS) was carried out in a UHV chamber equipped with an Omicron XPS (base pressure 5×10\textsuperscript{-10} Torr) with a monochromatic aluminum anode X-ray source of 1486.6 eV Kα radiation, all spectra were calibrated with the C1s peak at 284.6 eV as an internal standard. The $r_2$ relaxivity of NPs was determined using a 7.0-T MR scanner (Bio-Spec USR70/20, Bruker, Germany). The NPs were diluted with various iron concentrations in the range of 0–0.25 mM. $T_2$ relaxation time were obtained using $T_2$-map MSME sequence with the following parameters: TR/TE: 4,000/9.5 ms; echo image: 10; slice thickness: 0.5 mm; FOV: 2 × 2 cm; matrix: 256 × 256. The $r_2$ relaxivity was calculated by a linear fit of $1/T_2$ against iron concentrations. The PA imaging properties of euMel-Fe\textsubscript{3}O\textsubscript{4} NPs was studied by filled in EP tubes with series concentrations of aqueous NPs.

2.4 Photothermal effect of euMel-Fe\textsubscript{3}O\textsubscript{4} NPs
To access the photothermal effect of euMel-Fe$_3$O$_4$ NPs, 200 µL NPs aqueous solution with different iron concentrations (0–10 mM) were irradiated by 808 nm NIR laser at a power density of 2 W/cm$^2$ for 5 min. The euMel-Fe$_3$O$_4$ NPs solution at an iron concentration of 10 mM were also irradiated by 808 nm NIR laser at different power densities (0.5–2.5 W/cm$^2$) for 5 min. The solution temperature was recorded by an infrared thermal camera.

2.5 Cell culture

Human U-87 MG cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere with 5% CO$_2$. The medium was changed every other day. Cell viability was assessed by 0.4% trypan blue exclusion before experiments.

2.6 Cell viability assay

U-87 MG cells were seeded in 96-well plate with a density of 5000 cells per well and incubated overnight at 37 °C. euMel-Fe$_3$O$_4$ NPs with various iron concentrations were added into wells and incubated with cells for 24 h at 37 °C. After that, a standard MTT assay was carried out to determine the cell viability. Cell viability (%) was calculated as the absorbance percentage of untreated cells.

2.7 Cellular uptake assay

The uptake of euMel-Fe$_3$O$_4$ NPs by U-87 MG cells was visualized using Prussian blue staining. 2 × 10$^4$ U-87 MG cells were seeded in a 24-well plate and allowed to 70% confluence. Cells were then incubated with euMel-Fe$_3$O$_4$ NPs at an iron concentration of 10 µg/mL for 6 h. After washed with PBS (phosphate-buffered saline) twice and fixed with 4% PFA, cells were incubated in Perls staining solution for 20 min. After washed with deionized water twice and counterstained with nuclear fast red for 10 min, cells were examined under a microscope.

2.8 In vitro Cell MR/PA imaging

5 × 10$^4$ U-87 MG cells were seeded in a 6-well plate and allowed to 90% confluence. Then cells were incubated with euMel-Fe$_3$O$_4$ NPs at an iron concentration of 10 µg/mL or 20 µg/mL for 6 h. After washed twice with PBS, cells were collected and re-suspended in 200 µL of 1% low melting agarose. Cell MRI was performed using a 7.0 T scanner. T$_2$-weighted MSME sequences were performed with the parameters as follows: TR/TE: 4000/45 ms; matrix: 256 x 256; FOV: 40 x 40 mm$^2$; slice thickness: 1 mm; averages: 2. T$_2$ relaxation time was calculated using a single exponential fitting of the echo train. The PA images of cells were obtained at 800 nm using a PA system (Endra Nexus 128, Ann Arbor, MI).

2.9 In vitro photothermal therapy against U-87 MG cells

For cell viability assay, U-87 MG cells were incubated with euMel-Fe$_3$O$_4$ NPs at an iron concentration of 1 mM in 96-well plates. After 6 h, the cells were irradiated by an 808 nm NIR laser at a power density of 2 W/cm$^2$ for 10 min. Then, the culture media were replaced by fresh ones. After incubation for another 24 h, the cell viabilities were analyzed via the standard MTT assay.

2.10 Fluorescence microscopy imaging

U-87 MG cells were seeded in 6-well plates with a density of 2×10$^5$ cells per well and incubated overnight at 37 °C. euMel-Fe$_3$O$_4$ NPs were added into wells at an iron concentration of 1 mM. After 6 h, the cells were irradiated by an 808 nm NIR laser at a power density of 2 W/cm$^2$ for 10 min. Then, the culture media were replaced by fresh ones. After incubation for another 4 h, cells were co-stained with Calcein AM/PI for 30 min, washed with PBS for three times, and observed by a fluorescence microscope.

2.11 Tumor model

BALB/c nude mice (female, 4–5 weeks, 20 ± 2 g) were supplied by Center for Experimental Animals, Xiamen University, China. All animal experiments were performed in accordance with the protocol approved by the Committee for Animal Studies at Xiamen University. Tumor model was established by subcutaneous injection of 10$^6$ U-87 MG cells suspended in 80 µL PBS into the flank region of mice.

2.12 In vivo MR/PA dual-modal imaging

When the size of tumors reached to 6 mm in diameter, the U-87 MG tumor-bearing mice were intratumorally injected with Fe$_3$O$_4@$mel NCs (50 µL, 2 mg Fe/mL). For in vivo MRI, Turbo RARE-T$_2$ sequence were preformed using a 7.0 T small animal MR scanner (Bruker, German), with the imaging parameters as follows: TR/TE: 4,000/45 ms; averages: 2; slice thickness: 0.5 mm, FOV: 2.5 × 2.5 cm; matrix: 256 × 256. The signal intensity (SI) was measured with the same sized ROI on the same slice of T$_2$-weighted images. The change of SI was obtained using the following formula: $SI_{post} – SI_{pre}$)/(SI$_{pre}$ × 100%, where SI$_{pre}$ and SI$_{post}$ were the SI of tumor prior to and post injection of NCs, respectively. For in vivo PAI, the mice were imaged with a photoacoustic computed tomography scanner (Endra Nexus 128, USA).

2.13 In vivo tumor photothermal therapy

When the size of tumors reached to 6 mm in diameter, the mice were randomly divided into four groups (n=4 per group): no treatment (Group I), euMel-Fe$_3$O$_4$ NPs without laser treated (Group II), laser treated only (Group III) and euMel-Fe$_3$O$_4$ NPs plus laser treated (Group IV). 50 µL euMel-Fe$_3$O$_4$ NPs solution (2 mg Fe/kg) was directly injected into the tumor interstitium at multiple sites of tumor mass, enables effective distribution into surrounding tumor tissues. The tumors in group III and group IV were irradiated by 808 nm laser (2 W cm$^{-2}$, 10 min) at 1 h post-injection. The temperature at tumor site was recorded using an infrared thermal camera. For observation of in vivo photothermal therapy effect of euMel-Fe$_3$O$_4$ NPs, the tumor volume and body weight of mice were recorded every other day. The tumor volume (V) was measured using a caliper to calculate the tumor volume according to the formula: $V = \text{width} \times \text{length} \times 2$. Relative tumor volumes were calculated as $V/V_0$, where $V_0$ was the tumor volume when the treatment was initiated.

2.14 Histological analysis

The mice were sacrificed on day 15 after treatment, and the major organs including heart, liver, spleen, lung, kidney and tumor were harvested and fixed in 4% formalin for 24 h. The tissues were then paraffin embedded, sectioned into a thickness of 4 µm, and stained
with hematoxylin & eosin (H&E) and then observed using an optical microscope.

Fig. 1 Synthesis and characterization of euMel-Fe$_3$O$_4$ NPs. A) Illustration of the synthesis procedures of euMel-Fe$_3$O$_4$ NPs. B) TEM image of euMel-Fe$_3$O$_4$ NPs. C) HRTEM image of euMel-Fe$_3$O$_4$ NPs with crystalline planes indicated by arrows. D) SAED pattern of euMel-Fe$_3$O$_4$ NPs. E) Photograph showing euMel-Fe$_3$O$_4$ NPs aqueous solution after standing at room temperature for 24 h, and magnetic attraction for product collection. F) XRD pattern of euMel-Fe$_3$O$_4$ NPs. G) FT-IR spectra of euMel-Fe$_3$O$_4$ NPs. H) Distribution of the hydrodynamic diameter of euMel-Fe$_3$O$_4$ NPs determined by DLS. I) Surface zeta potential analysis of euMel-Fe$_3$O$_4$ NPs.

3. Results and discussion

3.1 Preparation and characterization of euMel-Fe$_3$O$_4$ NPs

The eumelanin-Fe$_3$O$_4$ hybrid nanoparticles (euMel-Fe$_3$O$_4$ NPs) was prepared by the one-step co-precipitation method with modifications (Fig. 1, A). The obtained euMel-Fe$_3$O$_4$ NPs aqueous solution was brownish black in color and well-dispersed in water (left), which was attracted to the side wall by a magnet for 10 min (right) (Fig. 1, E). After standing at room temperature for 24 h, the as-prepared NPs exhibited desired dispersibility in aqueous solution (Fig. 1, B), while the pristine Fe$_3$O$_4$ NPs were aggregated and precipitated (Fig. S1), suggesting the integration of eumelanin coating improved water-dispersibility of euMel-Fe$_3$O$_4$ NPs. The crystalline nature of euMel-Fe$_3$O$_4$ NPs was also demonstrated by detection of crystalline fringes. A typical cubic magnetite structure (311 plane) was observed through HRTEM image (Fig. 1, C) and SAED (selected area electron diffraction) pattern (Fig. 1, D) of euMel-Fe$_3$O$_4$ NPs. X-ray powder diffraction (XRPD) analysis further confirmed the presence of diffraction peaks indexed to spinel-structure of Fe$_3$O$_4$ (Fig. 1, F). These results are also in agreement with other studies relevant to Fe$_3$O$_4$ NPs. Besides, the functionality of euMel-Fe$_3$O$_4$ NPs was evidenced by FT-IR spectroscopy (Fig. 1, G). Which depicts a broad absorption at 3,420 cm$^{-1}$ (polymeric O-H groups), a twin-peaks range between 2,925—2,952 cm$^{-1}$ (stretching aliphatic C-H bonding), the symmetric COO$^-$ stretching vibrations at 1,586 cm$^{-1}$, and fingerprint regions between 1,450 cm$^{-1}$ and 650 cm$^{-1}$. Fe-O bond of Fe$_3$O$_4$ can be found at 600 cm$^{-1}$ and 580 cm$^{-1}$ (Fig. 1, G). DLS results showed that the hydrodynamic diameter (HD) of euMel-Fe$_3$O$_4$ NPs was 91.0 ± 24.5 nm (Fig. 1, H). The larger size examined by DLS than TEM observation was ascribed to the self-bridging of euMel-Fe$_3$O$_4$ NPs via eumelanin mediated π-π interactions and long-chain polymeric intertwine. The zeta ($\zeta$) potential analysis indicated that surface net-charge of NPs was -26.1 ± 4.5 mV (Fig. 1, I). Revealing that deprotonated carboxyl groups on the surface of euMel-Fe$_3$O$_4$ NPs contribute a negative charge. Additionally, the EDX profile and XPS spectra survey are in close relation with previously reported data (for details and discussion see Table S1†, Fig. S1-S7†).

3.2 Photothermal, MR $T_2$ and PA contrast profiles of euMel-Fe$_3$O$_4$ NPs

Considering that the wavelength of NIR laser irradiation used for photothermolysis and PA imaging studies is 808 nm, the absorbance
of euMel-Fe₃O₄ NPs in NIR region is one of the key factors to determine the photothermal conversion efficiency. UV–Vis absorption spectra were recorded after different iron concentrations of euMel-Fe₃O₄ NPs were dispersed in aqueous solutions (Fig. 2A). The pure water exhibited negligible absorption of the NIR light at 808 nm, while this absorption was continually enhanced as the iron concentrations increased. The most intense NIR light absorption was observed at 10 mM of euMel-Fe₃O₄ NPs. A linear correlation between absorbance and iron concentrations of euMel-Fe₃O₄ NPs was found using linear regression modeling (Fig. 2B). All these NIR light absorption results correspond to the increased temperature curves of euMel-Fe₃O₄ NP aqueous dispersion in Fig. 2C. As expected, higher amounts of euMel-Fe₃O₄ NPs provided greater temperature elevation with exponential increase from 30 °C to final temperatures at 35.0, 45.0, 52.5, 61.5 and 69.8 °C after 5 min NIR irradiation, when the euMel-Fe₃O₄ NPs concentrations were 0, 1.25, 2.5, 5 and 10 mM, respectively. Fig. 2D suggests that the range of temperature elevation was the most significant when the euMel-Fe₃O₄ NP concentration was 10 mM, which had a temperature elevation of 40 °C. Whereas water showed only 5 °C elevation. Clearly, this estimated that 10 mM of euMel-Fe₃O₄ exhibited the highest performance for photothermal treatment. The sample was then exposed to the 808 nm laser with different laser power densities (0.5, 1.0, 1.5, 2.0, 2.5 W cm⁻²) for 5 min, and the results showed that 2.5 W cm⁻² of 808 nm laser demonstrated highest temperature (from 30 to 74.4 °C) in 5 min (Fig. S8, S9††). However, the temperature differences between the sample at different laser power densities became smaller with the increase of power density, particularly between 2.0 and 2.5 W cm⁻² (Fig. S9, S10††), which could be due to the NIR energy overload of euMel-Fe₃O₄ NPs with concentration of 10 mM. In this regard, laser power density at 2.0 W cm⁻² was considered as suitable operating parameters for bio-application.

The magnetic behavior of euMel-Fe₃O₄ NPs was obtained by a vibrating sample magnetometer (VSM). The measurement of magnetization curve as a function of the magnetic field strength was performed. The absence of a hysteresis loop indicates zero coercivity, i.e. the superparamagnetic property of euMel-Fe₃O₄ NPs with a saturation magnetization (Ms) value of 47.8 emu/g (Fig. 2F). The magnetic behavior of euMel-Fe₃O₄ NPs was also compared with several commercially available IONPs from Guerbet (France), Ocean Nanotech (USA), and Oneder-hightech (China). At equivalent iron molar concentrations, euMel-Fe₃O₄ NPs outperformed other commercially available IONPs at the same concentration (Fig. 3A). Thus, the enhancement of T₂-weighted contrast performance may be partly attributed to the eumelanin integration. PA signal intensities among increased concentration of euMel-Fe₃O₄ NPs (0, 1.25, 2.5, 5 and 10 mM) was then examined via an PA system employing 800 nm excitation light. A positive linear correlation between PA signal intensity and euMel-Fe₃O₄ concentration was observed, and could be readily recognized from inserted images (Fig. S11††).

3.3 Characterization comparison with commercially available IONPs.

The material performance of euMel-Fe₃O₄ NPs was also compared with several commercially available IONPs from Guerbet (France), Ocean Nanotech (USA), and Oneder-hightech (China). At equivalent iron molar concentrations, euMel-Fe₃O₄ NPs had stronger absorption in the NIR region than those of the commercially available IONPs (Fig. 3A), which may be ascribed to the integration of eumelanin. The photothermal effects of solutions were determined by 808 nm NIR laser irradiation at 2 W/cm² for 5 min. As expected, the temperature elevation of euMel-Fe₃O₄ NPs has outperformed other commercially available IONPs at the same concentration (Fig. 3B). The final temperatures after 5 min were 68.8, 39.6, 64.0, and 52.4 °C, for euMel-Fe₃O₄ NPs and IONPs from Guerbet, Ocean Nanotech, and Oneder-hightech, respectively.

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NIR light absorption results (Fig. 3A) are consistent with the temperature curves in Fig. 2B. This indicates that the temperature enhancement was mainly induced by the strong NIR absorption of euMel-Fe₃O₄ NPs. In Fig. 3C, the T₂ relaxation rate measurements demonstrated that euMel-Fe₃O₄ NPs had a much higher relaxivity value than that of the commercially available IONPs. The T₂ relaxivity of euMel-Fe₃O₄ NPs and IONPs from Guerbet, Ocean Nanotech, and Oneder-hightech was about 245.88, 148.27, 198.91 and 95.95 mM \(^{-1}\)s\(^{-1}\), respectively. Furthermore, the comparison of the PA signal intensity between euMel-Fe₃O₄ NP and commercialized IONPs solutions has found that euMel-Fe₃O₄ NP exhibited excellent PA performance with signal intensity at 4240 ± 536, while commercialized IONPs solutions showed significant low signal intensity, range from 510 ± 43 to 525 ± 46 and 591 ± 38 (Fig. 3D).

3.4 Cytotoxicity, in vitro MR/PA property and photothermal treatment efficacy of euMel-Fe₃O₄ NPs

The high photothermal conversion efficiency and superior MR/PA signal profile of the euMel-Fe₃O₄ NPs prompted us to verify their applicability in vitro. To assess the in vitro MR/PA signal intensity of euMel-Fe₃O₄ NPs, human U-87 MG cells were used as a model of tumor tissue. The cells were incubated with euMel-Fe₃O₄ NPs at an iron concentration of 10 or 20 µg/mL for 6 h, and then T₂-weighted MSME sequences were performed using a 7.0 T scanner. The tested cells were divided into three groups, 1) untreated control, 2) 10 µg/mL of euMel-Fe₃O₄ NPs incubation, 3) 20 µg/mL of euMel-Fe₃O₄ NPs incubation. It was found that higher concentration of euMel-Fe₃O₄ NPs in cells exhibited much shorter T₂ relaxation time, revealing greater negative T₂-weighted contrast for MR imaging (Fig. 4A). This contrast effect can also be visualized from the inserts of Fig. 4A. Then, same procedure for comparison was performed for PA signal evaluation. Fig. 4B shows that cellular PA intensity was gradually increased up to 4-fold from 0 to 20 µg/mL of euMel-Fe₃O₄ NPs after incubation. Overall, both MR and PA cellular signal profile demonstrated desired contrast intensity for further applications.

As potential cytotoxicity is crucial for in vivo applications, we first evaluated the in vitro toxicity of euMel-Fe₃O₄ NPs towards human U-87 MG cells line. Prussian blue staining of U-87 MG cells in which incubated with euMel-Fe₃O₄ NPs were conducted to identify the iron contained NP internalization. This reveals that iron NPs can be found in cytoplasm of almost all of cells with euMel-Fe₃O₄ NP at 10 µg/mL concentrations (Fig. 4C). We then proceeded to evaluate the cell viability by incubated with different concentrations of euMel-Fe₃O₄ NPs for 24 h. The results of MTT assay from Fig. 4D demonstrates that only negligible cell death could be observed when the concentration euMel-Fe₃O₄ NPs in range of 0—100 µg/mL. The cell viability remained about 96.64% at high concentration of 100 ug Fe/mL, suggesting euMel-Fe₃O₄ NPs did not cause acute cytotoxicity and had a good safety profile for in vivo application. Moreover, the in vitro photothermalysis against U-87 MG cells was investigated. Cells were incubated with euMel-Fe₃O₄ NPs at an iron concentration of 1 mM for 6 h, followed by an 808 nm NIR laser irradiation at a power density of 2 W/cm² for 10 min. No obvious cellular death was detected among control groups, NPs only treated groups and laser only treated groups. While with the cells treated by both NPs and laser, the cell viability reduced notably at 43% as revealed by both the MTT assay and Calcein AM & propidium iodide (PI) co-staining assay under fluorescence microscopic observation (Fig. 4E and F).
Fig. 4 Cytotoxicity, in vitro MR/PA property and photothermal treatment efficacy of euMel-Fe$_3$O$_4$ NPs. A) Cell MR $T_2$ relaxation time of different concentrations of euMel-Fe$_3$O$_4$ NPs incubated with U-87 MG cells. B) PA signal intensities of different concentrations of euMel-Fe$_3$O$_4$ NPs incubated with U-87 MG cells. C) Prussian blue staining images of U-87 MG cells incubated with euMel-Fe$_3$O$_4$ NPs for 6 h. Scale bar = 50 μm. D) In vitro cell viabilities of U-87 MG cells treated with different concentrations of euMel-Fe$_3$O$_4$ NPs for 24 h. E) Viabilities of U-87 MG cells after incubation with PBS (control), euMel-Fe$_3$O$_4$ NPs (NPs only), PBS under laser irradiation (laser only) and euMel-Fe$_3$O$_4$ NPs under laser irradiation (NPs + laser). F) Fluorescence microscopy imaging at some condition of E) and co-stained with Calcein AM/PI. Error bars in D) and E) were based on the standard deviations (SD) of four parallel samples. Scale bar = 50 μm.

3.5 In vivo MR/PA dual-modal imaging-aided tumor photothermolysis

Encouraged by the high MR/PA contrast profile and robust photothermal effect of euMel-Fe$_3$O$_4$ NPs, we next applied euMel-Fe$_3$O$_4$ as an PA and $T_2$-MR contrast agent to investigate it’s in vivo imaging-assisted tumor photothermolysis. The U-87 MG tumor-bearing mice were scanned by an PA system employing 800 nm excitation light at pre- and post-interstitial injection with euMel-Fe$_3$O$_4$ (50 μL, 2 mg Fe/mL). Compared with pre-injected tumor (Fig. 5A), apparent signal contrast light up the whole tumor area after 5 min post injection and maintained the signal intensity within an hour (Fig. 5B, C). The results of quantitative ROI (region of interest) measurement illustrated that the relative signal intensity increased over time, from 1.0 ± 0.65 (pre-injection) to 7.9 ± 1.25 (5 min) and 9.6 ± 1.41 (1 h) (Fig. 5D), revealing the optimal imaging time point could be at any time within an hour after 5 min of post injection. Although PA imaging cannot provide whole-body imaging in contrast to conventional in vivo optical bioimaging, its relative deep penetration and high spatial resolution would be able to depict the detailed distribution of NPs inside the tumor tissue, and thus predict the therapeutic effect.

Relying on the high $r_2$ value of euMel-Fe$_3$O$_4$ NPs, same procedure was conducted for pre-treatment MR contrast imaging of tumor-bearing mice using the 7.0 T small animal MR scanner. A notable negative contrast effect after injection at 5 min and 1 h (Fig. 5F, G) could be observed when compared with pre-injection scan.
Accumulation of euMel-Fe$_3$O$_4$ was clearly displayed inside tumor with darkened $T_2$-contrast effect (yellow arrowheads in Fig. 5F, G). Similar to PA imaging, the relative negative signal intensity increased over time, which was reflected by decreased $T_2$-weighted signal intensity (Fig. 5H), from 103.0 ± 16.5 (pre-injection) to 87.1 ± 40.3 (5 min) and 54.6 ± 21.8 (1 h). These signal increments imply the gradual distribution of euMel-Fe$_3$O$_4$ NPs through the tumor interstitial space. MR imaging afforded sufficient spatio-temporal resolution for soft tissue examination, which can be used to retrieve the accurate location of euMel-Fe$_3$O$_4$ NPs inside tumor tissue. Taking both advantages of MR and PA imaging modalities, more precise information could be obtained for design and evaluation of therapeutic actions.

3.5 In vivo tumor photothermolysis

To evaluate their in vivo therapeutic potential, an aqueous solution of euMel-Fe$_3$O$_4$ NPs (50 μL, 2 mg Fe/kg) was injected into interstitium at multiple sites of U-87 MG tumor mass of xenograft model (tumors diameter ~ 6 mm), and tumors were then exposed to an 808 nm laser irradiation at 2 W cm$^{-2}$ for 10 min. Study subjects were randomly divided into four groups (n = 4 per group), 1) no treatment, 2) euMel-Fe$_3$O$_4$ NPs without laser treatment, 3) 808 nm laser only, and 4) euMel-Fe$_3$O$_4$ NPs with laser treatment. Fig. 6A shows the infrared thermal images of tumor surface under 808 nm laser irradiation with and without euMel-Fe$_3$O$_4$ NP administration. The presence of euMel-Fe$_3$O$_4$ achieved a rapid temperature elevation (initial temperature, 30.1 °C) of the tumor surface within 8 mins of post-injection, with final temperature reached at 51.3 °C (Fig. 6A). Whereas the control group (injected with PBS) showed only less than half quantity of temperature elevation (9.0 °C) in contrast to tumors after injection of euMel-Fe$_3$O$_4$ NPs (Fig. 6A). The results of tumor volume quantification demonstrate that tumors after injection of euMel-Fe$_3$O$_4$ followed by 808 nm laser treatment were completely eradicated without recurrence within 15 days (Fig. 6B, Fig. S13†). Conversely, tumor tissues of other groups did not exhibit any tumor inhibition tendency with statistical significance (Fig. 6B, Fig. S13†). The body weight of mice was recorded during period of time, the treated group showed no obvious changes in the body weight and was comparable to that of the laser only group, while other groups showed notable weight loss during the monitoring period (Fig. 6C). Therefore, our results verified the desired antitumor activity of the euMel-Fe$_3$O$_4$ NPs in treatment of U-87 MG tumor xenograft model.
same laser treatment condition as the control (laser only). B) Time-dependent tumor growth evaluation of the mice among different treatment groups: 1) Control group (PBS only), 2) euMel-Fe₃O₄ NPs without laser treatment, 3) 808 nm laser only, and 4) euMel-Fe₃O₄ NPs with laser treatment. C) The body weight changes of mice after different treatments. *P < 0.05 and **P < 0.01 were calculated using student’s t-test.

Finally, the histological analysis was performed to detect any tissue damage in major organs of tumor-bearing mice among different treatment conditions. The major organ slices including heart, liver, spleen, lung, and kidney were prepared and H&E stain was employed for characterization. No obvious morphological differences among major organ slices are observed between each group, and no noticeable morphology damage is found from each examined group (Fig. 7). Indicating no observable euMel-Fe₃O₄ NPs entered the blood circulation and retained in major organs. Based on the in vitro and in vivo results, euMel-Fe₃O₄ NPs upon interstitial delivery demonstrated no serious adverse effects but desired antitumor activity in small animal models. These results are in accordance with the previous conclusion that eumelanin and Fe₃O₄ NPs were found to be more promising because of their intrinsic characteristics of biocompatibility and biodegradability.

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Fig. 7 Micrographs of H&E stained major organ slices from mice with four groups of treatment conditions. Organs were collected after 15 days when the observation period was completed. No appreciable morphological abnormality was absorbed. Scale bar = 100 μm.

4. Conclusions

In summary, a biodegradable eumelanin-Fe₃O₄ hybrid nanoparticles (euMel-Fe₃O₄ NPs) with enhanced MR/PA imaging and effective photothermal properties, has offered opportunity for their theranostic application in treatment of glioblastoma xenograft model through intratumoral interstitial delivery. euMel-Fe₃O₄ is a polymer-crystal hybrid nanoparticle containing Fe₃O₄, numerous aromatic rings, hydroxyls and carboxyl groups, based on TEM, FT-IR, XPS and XRD analyses. The as-prepared euMel-Fe₃O₄ NPs exhibited excellent aqueous dispersity and magnetic responsiveness. euMel-Fe₃O₄ NPs were found to be virtually safe towards glial type cells. The histological examination of major organs (heart, liver, spleen, lung, and kidneys) from mice treated with an interstitial injection of euMel-Fe₃O₄ NPs were all found to be normal. Utilizing the superparamagnetic and NIR optical properties of euMel-Fe₃O₄ NPs, the injected dose and location of accumulation inside tumor mass were clearly visualized and quantified by MR/PA dual modal imaging. Such pre-treatment evaluation greatly assisted precise in vivo local NIR photothermolysis in treatment of glioblastoma. Moreover, the in-situ retention of these NPs could be gradually absorbed due to their excellent biocompatible and biodegradable compositions. Collectively, euMel-Fe₃O₄ NPs could be proposed as a potential class of theranostic nanoparticle with multiple imaging modalities as all-in-one nanomedicine.
Conflicts of interest

There are no conflicts to declare.

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Notes and references
