“All-in-One” Nanoparticles for Tri-modality Imaging-Guided Intracellular Photo-magnetic Hyperthermia Therapy under Intravenous Administration

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Abstract: Great efforts have been devoted so far to combine nano-magnetic hyperthermia (NMH) and nano-photothermal therapy (NPTT) to achieve encouraging additive therapeutic performance in vitro and in vivo with limitation to direct intratumoral injection and no guidance of multimodality molecular imaging. In this study, we developed a novel multifunctional theranostic nanoplatform (MNP@PES-Cy7/2-DG) consisting of magnetic nanoparticles (MNPs), poly(3,4-ethylenedioxythiophene):poly(4-styrenesulfonate) (PES),
Cyanine7 (Cy7) and 2-Deoxyglucose-PEG (2-DG). And then we applied them for combined photo-magnetic hyperthermia therapy under intravenous administration which was simultaneously guided by tri-modality molecular imaging. Remarkably, nanoparticles were found aggregated mainly in the cytoplasm of tumor cells in vitro and in vivo, and exhibited stealth-like behavior with a long second phase blood circulation half-life of 20.38 ± 4.18 h. Under the guidance of photoacoustic/near-infrared fluorescence/magnetic resonance tri-modality imaging, tumors could be completely eliminated under intracellular photo-magnetic hyperthermia therapy with additive therapeutic effect due to precise hyperthermia. This study may promote a further exploration of such platform for clinical application.

1. Introduction

Nanoparticle-mediated hyperthermia, such as nano-magnetic and nano-photothermal, has drawn tremendous attention due to its high therapeutic efficiency and selective local treatment with minimum adverse effects on collateral tissues.[1-3] Nano-magnetic hyperthermia (NMH) has been clinically tested as an effective treatment in various human cancers with direct injection of magnetic iron oxide nanoparticles into the solid tumor.[4-9] In nano-photothermal therapy (NPTT), optical absorbing nanomaterials are employed to efficiently convert light into localized heat energy for cancer cell death and tissue destruction.[10-11] However, both NMH and NPTT still suffer from certain disadvantages. The need for high magnetic nanoparticles (MNPs) concentration and the significant reduction of heating efficiency in the cellular environment remain in their clinical applications of
Additionally, the drawbacks of high doses of laser irradiation and inevitable depth-dependent decline of laser intensity have greatly hindered the clinic translation of NPTT.\(^{[14]}\) Recently, great efforts have been devoted to combine NMH and NPTT with the aim to achieve synergistic therapeutic performance with attenuated concentration and safe light power.\(^{[15-17]}\) Unfortunately, the photo-magnetic hyperthermia can be performed only through direct intratumoral injection owing to low heating efficiency of the nanoparticles, tremendously limiting the realization of precise nanoparticles-mediated hyperthermia owing to inhomogeneous distribution of the NPs and undesired uptake by phagocytic cells not tumor cells in tumor site.\(^{[17-19]}\) Therefore, it is still urgent to develop new multifunctional nanocomposites for realizing real intracellular photo-magnetic hyperthermia \textit{in vivo} by intravenous administration.

In nanoparticle-mediated hyperthermia, of particular interest is the engineering of multifunctional nanotheranostic platforms that combine imaging and therapy together to allow to simultaneously diagnose and treat diseases, track the agent’s location, and evaluate the treatment efficacy.\(^{[20-21]}\) Among various imaging modalities, magnetic resonance imaging (MRI) is widely used in clinics and can provide an independent imaging with high soft tissue contrast.\(^{[22]}\) Photoacoustic imaging (PAI) that hybridizes the merits of optical and acoustic imaging particularly specializes in diagnosis of tumor pathophysiological status with high spatial resolution and deep penetration, which, nevertheless, has the disadvantage of small imaging field of view.\(^{[23-24]}\) Fluorescence molecular imaging (FMI) can reveal pharmacokinetic and biodistribution in the whole body in real-time with high-sensitivity and
high-specificity, but suffers from low spatial resolution and poor tissue penetration.\cite{25}

Therefore, not only can the combination of PAI, MRI, and FMI integrate their various advantages and overcome intrinsic limitations, but also provide accurate and comprehensive diagnostic information to guide hyperthermia. However, the complex synthesis and application of multifunctional nanocomposite to integrate all ingredients together (“all-in-one”) are still formidable challenges.

Very recently, it has been found that near-infrared (NIR)-absorbing conjugated polymers with high photothermal conversion efficiency could serve as excellent NPTT agent, showing good biocompatibility and promising results in cancer cell laser ablation \textit{in vitro} and \textit{in vivo}.\cite{26} Poly(3,4-ethylenedioxythiophene):poly(4-styrenesulfonate) (PEDOT:PSS, or PES), a conductive polymer mixture with strong NIR absorbance, has been successively used for \textit{in vivo} photothermal treatment of cancer with high efficiency.\cite{27-29} MNP@PEDOT:PSS core-shell nanoparticles fabricated by our group have shown balanced, prominent magnetic-optical properties in suspension,\cite{30} making it possible for photo-magnetic hyperthermia therapy of cancer. With MNP@PES core-shell backbones, the other imaging and targeting functions could be facile prepared with a well-defined design while maintaining high therapeutic efficiency. Inspired by this intriguing property, we established in the present study a multi-shell structure in which the NIR dye Cyanine7 (Cy7) and the targeting agent 2-Deoxyglucose-PEG (2-DG) were attached on the surface of core-shell MNP@PES nanoparticles. Encouraged by the excellent photo-magnetic properties in solution, favorable biocompatibility \textit{in vitro} and dramatically long blood circulation \textit{in vivo}, we delivered the
multifunctional nanoprobes to the breast tumor site by intravenous injection for additive photo-magnetic hyperthermia under the guidance of multimodality molecular imaging as showed in Scheme 1. Interestingly, we observed largely intracellular localization of nanoprobes in cytoplasm of tumor cells and additively therapeutic effects for photo-magnetic hyperthermia *in vitro* and *in vivo*. Furthermore, we found that the nanocomposites could also simultaneously facilitate the three imagings: whole-body tumor localization using MRI, high spatial resolution and local three-dimensional imaging using PAI, and high-sensitivity, high-specificity and real-time imaging using NIR fluorescence imaging. Under the guidance of multimodality imaging, tumors were completely eliminated under intracellular photo-magnetic hyperthermia therapy. Our results encourage further exploration of other magneto-NIR absorbing nanocomposites for more precise nanoparticle-mediated hyperthermia.
Scheme 1. Schematic illustration of tri-modality molecular imaging-guided intracellular photo-magnetic hyperthermia therapy under intravenous administration.

2. Results and Discussion

2.1. Synthesis and Characterization of MNP@PES-Cy7/2-DG

Figure 1a schematically illustrates the key steps in the synthesis of the MNP@PES-Cy7/2-DG nanoparticle. After synthesizing \( \text{Fe}_3\text{O}_4 \) nanoparticles following a solvothermal method,\(^{[31]} \) \( \text{Fe}_3\text{O}_4@\text{PEDOT:PSS} \) nanocomposites were synthesized by an \textit{in situ} surface oxidative polymerization technique according to our previously developed protocol.\(^{[30]} \) The synthesized \( \text{Fe}_3\text{O}_4 \) cores were about 30–35 nm and good single crystallization in the cubic phase (Figure S1a, b, c, d). The MNP@PES had an average diameter of 30-40 nm and showed a highly uniform morphology from the transmission
electron microscopy (TEM) images in Figure 2b. The high-resolution TEM (HRTEM) images in Figure 2c further showed that the PEDOT:PSS (PES) nanoshell with a uniform thin structure and a thickness of ~3 nm on average. Energy dispersive X-ray spectroscopy (EDS) mapping and scanning transmission electron microscopy (STEM) further confirmed the core-shell structure and the polymer coating layer (Figure 2d).

We then functionalized the MNP@PES following Liu’s LBL polymer coating method. After step-by-step modified by a cationic polymer poly-allylamine hydrochloride (PAH) and anionic polymer PAA respectively, MNP@PES nanocomposites were added with a certain amount of amine-2-DG (targeting cancer cells) and anime-cyanine7 dyes (or anime-FITC), which conjugating with PAA via amide formation after N-(3-dimethylaminopropyl-N’-ethylcarbodiimide) hydrochloride (EDC) was added, yielding MNP@PES-Cy7/2-DG or MNP@PES-FITC/2-DG. The composition was approximately MNP@PES: PAH: PAA: PEG-2-DG: Cy7 (or FITC) = 1: 2: 2: 1: 0.01 by weight.

The hydrodynamic sizes of nanoparticles increased from about 60 nm to 94 nm as the 2-DG layer of polymer coatings was added (Figure S2). Moreover, the zeta potential of these nanoparticles (Figure S3) changed from -20 mV to +35 mV after PAH encapsulation, and finally returned to -18 mV as amine-2-DG was conjugated to surface carboxyl groups on the nanoparticles. These results indicated the successful coating of 2-DG on the outer layer of MNP@PES. After 2-DG modification, the NPs exhibited significantly improved stability in various physiological solutions such as water, phosphate buffered saline (PBS), fetal bovine serum, and H-DMEM cell medium (Figure S4a). Moreover, the multifunctional
MNP@PES-Cy7/2-DG nanoparticle was very stable even under shaking for 72 h at 37 °C (Figure S4b).

Figure 1. Synthesis and characterization of MNP@PES-Cy7/2-DG. (a): A schematic diagram of the fabrication process; (b): TEM image of the MNP@PEDOT:PSS nanocomposite; (c): high-resolution TEM image; (d): EDS mapping images, scale bar represents 20 nm; (e) UV-Vis-NIR spectrum of three nanoparticles; (f) Photoluminescence (PL) spectra of different NPs at same concentration with the pulsed laser excitations at 750 nm; (g) Room temperature magnetization curve of the nanoparticles.

The magneto-optical properties of the MNP@PES-Cy7/2-DG nanoparticles were examined. Based on the UV-vis-NIR absorption spectrum, MNP@PES exhibited high NIR absorption from 700 to 1100 nm with a peak at about 800 nm (Figure 1e).
photoluminescence (PL) spectra of MNP@PES-Cy7/2-DG showed obvious emission at
~790nm (Figure 1f) or MNP@PES-PEG/FITC at ~525nm (for cell experiment) in Figure S5.
MNP@PES NPs also possessed super-paramagnetism and a relatively high saturation
magnetization (Ms) of approximately 76 emu/g (Figure 1g), suitable for application to
MRI. The MNP@PES-Cy7/2-DG NPs were anticipated to be qualified for PA imaging in
suspension solution. As expected, the PA signal increased substantially as the NP
collection increased and was linearly correlated with the concentration, indicating that
MNP@PES-Cy7/2-DG is a promising candidate for PAI (Figure S6). The MRI signal of
MNP@PES was equally striking according to our previous work, while the estimated
transverse relaxivity ($r_2$) was 132.97 mM$^{-1}$S$^{-1}$. The balanced, excellent magnetic-optical
properties indicated that MNP@PES-Cy7/2-DG NPs have great potential for multimodality
imaging and photo-magnetic hyperthermia.

2.2. Photo-magnetic hyperthermia in Suspension Solution

Encouraged by the excellent magnetic-optical properties of MNP@PES-Cy7/2-DG
nanoparticles, photo-magnetic hyperthermia was examined in a suspension solution. We
developed an equipment for photo-magnetic hyperthermia, as shown in Figure 2a. Samples
(solution, cells, or mice) were placed in the middle of the coil, the laser and alternating
magnetic field (AMF) were adapted synchronously, and the temperature increase was
recorded using an infrared thermal imaging (IR) camera located at the end of the coil cavity.
Figure 2. Photo-magnetic hyperthermia in aqueous suspension. (a): Scheme of the experimental device for combined hyperthermia experiments, consisting of a magnetic coil in which the sample is placed so that it can be stimulated by the near-infrared (NIR) laser (808 nm). The temperature increase was recorded using an infrared thermal imaging (IR) camera located at the end of the coil cavity; (b): Heating curves of MNP@PES-Cy7/2-DG solutions at various concentrations under 808 nm laser irradiation at a power density of 0.75 W/cm²; (c): Heating curves of suspensions of nanocomposites at various concentrations in an alternating magnetic field (200 kHz, 38 kA/m); (d): Panel of thermal images acquired by the IR camera on samples at three concentrations; (e): Average temperature...
increase recorded for nanocomposites under the three heating protocols. Data were given as mean ± SD (n = 3).

Under 808 nm NIR laser irradiation at a power density of 0.75 W/cm², the solutions showed excellent photothermal effects with obvious concentration-dependent temperature increases (Figure 2b). Additionally, the aqueous suspensions of MNP@PES-Cy7/2-DG NPs also showed an obvious concentration-dependent temperature increase for NMH and exhibited a decent hyperthermia effect (Figure 2c). The SLP (the specific loss power) obtained was 704.5 W/g with NMH alone and 2371.5 W/g with NPTT alone, respectively, corresponding to previous studies for stand-alone NMH or NPTT.¹⁷, ²⁶

In order to combine with three-mode hyperthermia, three heating protocols were selected: (i) NMH-mode, AMF of 200 kHz and 38 kA m⁻¹ for 10 min; (ii) NPTT-mode, 808 nm NIR laser at a power density of 0.75 W/cm² for 10 min; and (iii) Dual-mode, the simultaneous application of both AMF and laser irradiation for 10 min. For the Dual-mode, the temperature increased 24.2 °C, 30.8 °C, and 40.7 °C for 200 μg/mL, 500 μg/mL, and 1 mg/mL, respectively, after 10 min, which approximately matched the sum of increases for the two individual modalities (Figure 2d and Figure 2e). This accumulation of hyperthermia effects was also observed for 300 μg/mL, 400 μg/mL, and 700 μg/mL (Figure S7 and Figure 2e).

In brief, the MNP@PES-Cy7/2-DG nanoparticles showed excellent photo-magnetic heating performance that efficiently combined the magnetic hyperthermia potential of MNP
cores and the photothermal effects of PES shell. Additionally, the heating of photo-magnetic hyperthermia equaled the sum of the heating observed for NMH or NPTT alone.

2.3. Cytotoxicity, Cellular Uptake and Combined Hyperthermia In Vitro

Before we tested photo-magnetic hyperthermia using our MNP@PES-Cy7/2-DG nanocomposite in vitro, we first evaluated the cytotoxicity of NPs. MCF-7 human breast cancer cells and L929 murine fibroblast cells were treated with various concentrations of nanoparticles for 24 h and 48 h. The MNP@PES-Cy7/2-DG nanocomposite exhibited little cell toxicity even at 200 μg/mL after 48 h of incubation (Figure 3a).
Figure 3. *In vitro* cell culture experiments. (a), Cell viability data obtained from the CCK8 assays of L929 cells and MCF-7 cells incubated with MNP@PES-Cy7/2-DG nanocomposite at different concentrations; (b). Confocal laser scanning microscopy (CLSM) images of MCF-7 cells treated for 4 h with different NPs (control group without NPs, NPs-PEG, and NPs-2-DG), cytoskeleton was stained with Rhodamine B (red) -labeled phalloidin (Sigma, USA), the nucleus was counterstained with (blue) 4', 6-diamidino-2-phenylindole (DAPI, Sigma, USA), and the NPs grafted with FITC were green, the scar bar represents 20 um; (c), Corresponding panels of thermal images acquired inside the coil setup by the IR camera are shown below for NMH, NPTT and NMH+NPTT; (d), Relative viability of MCF-7 cells under the three different heating protocols; (e) Fluorescence microscopy image of MCF-7 cells, stained with PI (red), stained with Calcein-AM (green), and a merged image under the three different heating protocols, the scar bar is 100 um; (f), FACS analysis of MCF-7 cells after different treatments for the following conditions: NP concentration 200 μg/mL, power density of the 808 nm laser of 0.75 W/cm², alternating field conditions of 200 kHz and 38 kA/m. Data were given as mean ± SD (n = 5); **p < 0.01.

The intracellular uptake and distribution of MNP@PES-dye/2-DG NPs (replacing Cy7 with FITC for cellular imaging in visible light) were investigated in MCF-7 breast cancer cells. These cells were incubated with different kinds of NPs for 4 h and stained with DAPI for nuclei (blue) and Rhodamine B for cytoskeleton detection (red). Compared with the weak green fluorescence observed in the control and NPs-PEG groups, much stronger green fluorescence was detected inside the cells for the NPs-2-DG group (Figure 3b and Figure S8a), indicating the cellular uptake efficacy by MCF-7 cells for MNP@PES-dye/2-DG NPs was superior to that for NPs-PEG. The targeting effect of 2-DG was further confirmed by mouse L929 fibroblast cells and mouse bone marrow derived macrophage RAW 264.7 (Figure S8b). There was weak fluorescence observed in the cytoplasm of L929 cells but relatively strong fluorescent signal in the macrophages. Additionally, the average intensities of NPs for different cells further indicated the uptake of 2-DG-NPs by tumor cells was appreciably greater than that of macrophages (Figure S9). The substantial increase in NP...
endocytosis by MCF-7 cells after 2-DG modification is mainly explained that tumor cells frequently exhibit increased glucose consumption compared to normal cells, therefore, particularly attract NPs with surface coating of glucose analogues 2-DG according to previous work. Moreover, significant internalization of MNP@PES-dye/2-DG NPs within the tumor cells provides substantial support for the feasibility of cellular inside-out photo-magnetic hyperthermia, as will be discussed later.

To quantitatively evaluate the heating effect at the cellular level, about 5 million cells labeled with MNP@PES-dye/2-DG were digested, placed in an Eppendorf tube, and subjected to three heating protocols described in the suspension analysis above. NMH was obviously less efficient at the cellular level than in suspension; the temperature change declined from 9.2 °C to about 3 °C. In comparison, NPTT was not hindered by cellular confinement (resulting in a 15°C increase both in cultured cells and in suspension). Remarkably, the heating effect of photo-magnetic hyperthermia was additive, resulting in a 22 °C increase higher than the sum of NMH-mode and NPTT-mode. It is possible that the thermal energy provided by laser stimulation frees the magnetic nanoparticles (~35 nm, Figure 1a) and restores their Brownian motion (Brownian relaxation is generally prominent for larger particles) in the cellular environment according to previous work.

We quantitatively and qualitatively evaluated the effect of photo-magnetic hyperthermia by CCK8 assays and staining with Calcein-AM/PI. Particularly, the dual-mode basically killed all cells at 200 μg/mL, while the NMH-mode and NPTT-mode only killed a portion of the MCF-7 cells (Figure 3d, Figure S10). By staining with Calcein-AM and PI to detect
apoptosis, extensive cell death was observed after incubation with MNP@PES-Cy7/2-DG NPs under the dual-mode (Figure 3e). However, minimal apoptosis was observed for cells treated with NMH, NPTT, or laser and AMF only (Figure S11), and MCF-7 cells showed no apoptosis after treatment with only NPs, in agreement with the CCK8 results. Finally, the effects of photo-magnetic hyperthermia therapy on MCF-7 cells were further quantitatively evaluated by flow cytometry (FACS, Figure 3f and Figure S12). Remarkably, more than 96% of the MCF-7 cells were apoptotic for photo-magnetic hyperthermia while only a small amount of apoptosis of MCF-7 cells for NPTT and NMH, and the majority of cells still survived for five control groups (Laser only, AMF only, NPs only, AMF plus Laser, and the control).

These in vitro results not only showed that the MNP@PES-Cy7/2-DG NPs could significantly accumulate in tumor cells, but also confirmed the photo-magnetic hyperthermia of MNP@PES-Cy7/2-DG NPs could more effectively exterminate cancer cells under relatively mild conditions. Moreover, the heating effect of combined hyperthermia for MNP@PES-Cy7/2-DG NPs was additive (or, to some extent, synergistic).

2.4. Pharmacokinetics, Biodistribution and Triple-modality Imaging In Vivo

Inspired by the promising results in aqueous solutions and in vitro, we utilized MNP@PES-Cy7/2-DG NPs as a multifunctional platform for multimodality molecular imaging of tumor-bearing mice. All the experiments involving animals were performed according to the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Tsinghua University.
First, the BALB/c mice bearing MCF-7 tumors were intravenously injected with MNP@PES-Cy7/2-DG NPs (0.15 mL, 0.5 mg/mL) for the biodistribution examinations. The pharmacokinetics profile of the NPs was examined by measuring the fluorescence intensity of Cy7 to determine the concentrations in blood at different time intervals post-injection (Figure 4a). Blood circulation of the MNP@PES-Cy7/2-DG NPs showed the typical two compartment model: the first phase (distribution phase) with a half-life of only 1.84 ± 0.23 h, the long second phase (elimination phase, the process for NPs clearance) with a half-life of 20.38 ± 4.18 h. The long blood circulation of the MNP@PES-Cy7/2-DG NPs can effectively reduce the phagocytosis of macrophages and favor the enhanced EPR effect.\textsuperscript{35}
Figure 4. Pharmacokinetic and biodistribution analysis. (a) Blood circulation curve of the MNP@PES-Cy7/2-DG determined by measuring the Cy7 fluorescence intensity in the blood of the mice at different time points after injection. (b) In vivo NIR fluorescence images of the NPs-injected mice at different time points. (c) Ex vivo fluorescence images of the tumor and major organs at 24 h post-injection. T, tumor; H, heart; Li, liver; K, kidney; S, spleen; Lu, lung. (d) Prussian-blue stained tissue slices of major organs collected from mice 24 h after intravenously injected with NPs, bars represent 50 μm. (e) Quantitative biodistribution analysis of the NPs in mice by measuring the Cy7 fluorescence intensity in the major organs and tumors at three different time points. (f) TEM images
the ultrastructural characteristics of the tumor tissue 24 h after intravenous injection. Nanoparticles were mainly localized within tumor cells (left), instead of phagocytic cells (right) or collagen fibers (right). N and C represent nucleus and cytoplasm, respectively. Data were given as mean ± SD (n = 4); *p < 0.05.

Then, the biodistribution of NPs was directly observed by the NIR fluorescence imaging in real-time as showed in Figure 4b. Considerable fluorescence could be observed from 4 h post-injection and gradually reached its maximum at 24 h post-injection, indicating the continuously accumulation of NPs at the tumor site, and then decreased until 48 h post-injection. The signal to background ratio (SBR) increased by about 3.2 times at 24 h post-injection compared to the intensity before injection, and maintained at a relatively high level in the following 24 h (Figure S13a and Figure 4b), suggesting good retention in the tumor. Ex vivo fluorescence images of 24 h post-injection (Figure 4c) further conformed enrichment of NPs in tumor and some metabolic organs such as liver and spleen owing to the reticuloendothelial system (RES). Moreover, micro-organizational examination of a tumor with Prussian blue staining of iron highlighted the significant colocalization of MNP@PES-Cy7/2-DG nanoparticles in the tumor section (Figure 4d). Furthermore, a quantitative biodistribution analysis of the MNP@PES-Cy7/2-DG nanoparticles in mice was conducted (Figure 4e). A group of tumor-bearing mice was scarified at 12, 24, and 48 h post-injection and the fluorescence intensity of Cy7 in the major organs of mice and tumors were analyzed by fluorescence spectrophotometer after the homogenized tissues were dissolved in the lysis buffer. At 24 h post-injection, NPs were largely distributed in tumor and reticuloendothelial (RES) organs such as liver, spleen as consistent with the above ex vivo fluorescence examination.
Furthermore, TEM images of ultrastructural characteristics of the tumor tissue 24 h after intravenous injection showed the nanoparticles were mainly localized within tumor cells instead of phagocytic cells or collagen fibers (Figure 4f). On the contrary, nanoprobes were confined and localized in phagocytic cells or collagen fibers following intratumoral injection in previous work.\cite{17} Therefore, the significant internalization of MNP@PES-dye/2-DG NPs within the tumor cells provides substantial support for the feasibility of cellular inside-out photo-magnetic hyperthermia \textit{in vivo}.

![Figure 5. In vivo MRI and PA imaging.](image)

Apart from NIR fluorescence imaging, MR and PA imaging were further carried out by using the MNP@PES-dye/2-DG NPs as the contrast agent owing to the excellent optical-magnetic properties. \textit{T}_2-weighted MR images of MCF-7 breast-tumor-bearing mice...
showed obvious $T_2$-weighted contrast 24 h after injection (Figure 5a), consistent with the FMI results. For PAI, the tumor tissue was distinctly visualized 24 h after injections (Figure 5b), further demonstrating the high tumor uptake of NPs. Specifically, The MR images from after the injection showed good localization of tumor in the whole body with high spatial resolution, and demonstrated clear visualization of the tumor with excellent soft tissue contrast (Figure 5a). PA images from after the injection provided three-dimensional imaging in a small tumor region while maintaining rich optical contrast, high spatial resolution and depth (Figure 5b), clearly showing the distribution of blood vessels in tumors and the pharmokinetics of the probes. Our results demonstrate that MNP@PES-Cy7/2-DG is an effective multifunctional probe for synchronous tri-modality molecular imaging that can provide more comprehensive diagnosis information.

2.5. Photo-magnetic Hyperthermia Therapy In Vivo

The additive hyperthermia effect of the MNP@PES-Cy7/2-DG nanocomposite based on in vitro experiments together with the high tumor accumulation of those nanoparticles uncovered in our imaging experiments inspired us to evaluate the photo-magnetic hyperthermia treatment on MCF-7 breast-tumor-bearing mice in vivo. Thirty female mice were randomly divided into five groups (n = 6 per group): a NMH + NPTT group, NMH group, NPTT group, Physical irradiation group (AMF + Laser), and a control group injected with PBS without irradiation. At 24 h after injection (20 mg/kg for each mouse) when maximum NPs accumulated in tumors, AMF or laser radiation was applied at the tumor site, and the surface temperature was synchronously monitored using an IR camera (Figure 6a).
The temperature in the tumor reached 34.5 °C (~4.5 °C increase) and 44 °C (~14 °C increase) for NMH and NPTT alone, respectively, and 52 °C (~22 °C increase) for photo-magnetic hyperthermia treatment (Figure 6a and Figure S14), while the AMF + Laser group only exhibited a minor temperature increase (~1 °C increase). These thermal imaging results showed the photo-magnetic hyperthermia therapy amplified the heating in vivo, and the effect of temperature increase was consistent with the additive effect for combined hyperthermia in vitro.

Figure 6b shows representative images from five groups before treatment and at day 35 after treatment. The tumors in the photo-magnetic group were completely burnt, leaving black scars at the original tumor sites. In comparison, the tumors in the NMH group and NPTT group grew to varying degrees, and the tumors in control group and AMF + Laser group exhibited more substantial growth. The tumor volumes and body weights were then measured at an interval of 2 days. As shown in Figure 6c, a remarkable decrease in tumor volume was observed from the third day in the NMH + NPTT group, and from the ninth day, tumors were completely eliminated, without relapse after 35 d. In mice administered NPTT or NMH, tumors were slightly inhibited in the first 7 days, but subsequently recrudesced, whereas the other control groups showed sustained and rapid tumor growth. Moreover, the changes in body weight were consistent with the relative tumors changes (Figure 6d), and the group treated with photo-magnetic hyperthermia had the largest average weight. MR images and PA images obtained before and 35 days after treatment demonstrated that tumors in the NMH + NPTT group disappeared (Figure 6e and Figure S16), whereas the tumors in the
NMH and NPTT groups increased dramatically. Moreover, we analyzed the behaviors of MNP@PES-Cy7/2-DG-injected mice after hyperthermia treatment, and did not observe obvious toxic effects over 35 days. Mice were sacrificed on day 35 for H&E staining, and no significant abnormalities in major organs were observed (Figure 6f and Figure S17). These results indicated MNP@PEDOT-Cy7/2-DG nanoparticles can completely eliminate breast tumor in additive photo-magnetic hyperthermia by intravenous injection in vivo without other side effects.

**Figure 6.** *In vivo* photo-magnetic hyperthermia of MCF-7 breast-tumor-bearing mice for combined NMH and NPTT. (a), IR thermal images of groups under four different heating protocols; (b)
Representative images of different groups before and 35 days after treatment; (c) Growth of MCF-7 tumors in different groups of mice after various treatments (tumor volumes were normalized against their initial sizes). Error bars represent standard deviations; (d) Body weights of mice in different groups after various treatments; (e), (D) $T_2$ MR images of mice before and 35 days after different hyperthermia treatments; (f), H&E-stained images of major organs collected from control and NMH+NPTT groups; bars represent 100 μm. Data were given as mean ± SD (n = 6); **$p$ < 0.01, ***$p$ < 0.005.

3. Conclusions

In summary, we have successfully fabricated multifunctional MNP@PES-Cy7/2-DG nanoparticles with excellent photo-magnetic properties, favorable biocompatibility and dramatically long blood circulation. And then we applied them to tri-modality molecular imaging guided additive photo-magnetic hyperthermia that combined NMH and NPTT under intravenous injection, achieving complete cell death in vitro and tumor ablation completely in vivo. Moreover, Amplifying or additive heating effects of photo-magnetic hyperthermia ensured effectively intravenous treatment of tumor by photo-magnetic hyperthermia.

Interestingly, we observed largely intracellular localization of nanoprobes in cytoplasm of tumor cells in vitro an in vivo. Furthermore, combining with tri-modality molecular imaging with unique features could provide more comprehensive diagnosis information. Both of this provide substantial support for the feasibility of more precisely intecellular inside-out photo-magnetic hyperthermia. In particular, the complete tumor ablation could not be achieved by using exclusively magnetic hyperthermia only or doubling the injected dose, mainly because of the relatively low SLP values of magnetic nanoparticles. Even with high efficiency and more simplicity, fluorescent measurements are relatively less accurate comparing with ICP-MS and gamma counting for quantifying nanoparticle concentration in
tissues. In brief, our work not only demonstrates composite MNP@PEDOT-Cy7/2-DG nanoparticles are ideal theranostic nanoplatforms for multimodality imaging guided intracellular photo-magnetic hyperthermia therapy, but also promise the developing of other types of nanocomposites with multifunctional, efficient, and safe treatments in more effective clinical application of precise hyperthermia.

4. Experimental Section

Synthesis of MNP@PES-Cy7/2-DG: First, Fe₃O₄ nanoparticles of approximately 30-35 nm were obtained using a one-pot solvothermal method following our previous methods[31] in an ethylene glycol/diethylene glycol (EG/DEG = 1:5) binary system.

The Fe₃O₄@PEFOT:PSS nanoparticles were synthesized by an in situ surface polymerization method[30]. Briefly, 48.6 mg of Fe₃O₄ nanoparticles and 519 mg of sodium dodecyl sulfate (SDS) were mixed in 225 mL of water by ultrasonication for 30 min, and the pH was adjusted to about 1-2. Next, 3,4-ethylenedioxythiophene (EDOT) and polystyrene sulfonic acid sodium (NaPSS) (molar ratio, EDOT: SO₃⁻ = 1:2) were homogeneously dispersed in water, added to the Fe₃O₄-SDS solution, and stirred for another 4 h. Then, 250 mL of APS (1 mg/mL) was added dropwise to the solution and stirred constantly for 10 h. The resulting black product was collected and washed with distilled water several times and then re-dispersed in 100 mL of DI water (~0.5 mg/mL).

The synthesized Fe₃O₄@PEFOT:PSS nanoparticles were negatively charged and modified using a LBL method by electrostatic interaction, Polyallylamine hydrochloride (PAH; 10 mg) was dissolved in 5 mL of water and added dropwise to 10 mL of Fe₃O₄@PEFOT:PSS solution. After ultrasonication and stirring simultaneously for 5 h, the solution was purified by filtration through molecular weight cut-off (MWCO) filters (120 kDa) to remove excess PAH. Then, the MNP@PES/PAH solution was added dropwise to 5 mL of polyactic acid (PAA) solution (2 mg/mL), ultrasonicated for 30 min, and stirred for 5 h. The solution was purified by filtration through 120 kDa MWCO filters again. After adjusting the pH to about 7–8, 10 mg of N-(3-dimethylaminopropyl-N'-ethylcarbodiimide) hydrochloride (EDC) was added to the mixture to induce cross-linking between PAH and PAA overnight. Samples were then purified by filtration through 120 kDa MWCO filters and re-dispersed in 10 mL of water.

Finally, 5 mg of NH₂-PEGn-GLUC (M_w = 3500) and 50 μg of dye (NH₂-Cy7 or NH₂-FITC) were dissolved in 1 mL of dimethyl sulfoxide (DMSO) and supplemented with 10 mL of the MNP@PES/PAH/PAA solution. After vortexing for 30 min, EDC (2 mg/mL) dissolved in 5 mL of

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DMSO was quickly added to the NP solution and reacted for 12 h in the dark with continuous mixing. The final particles were purified by magnetic separation and centrifuged at 15,000 × g for 30 min (3×).

**Characterization:** For transmission electron microscopy (TEM), the Hitachi HT7700 (Tokyo, Japan) was used. High-resolution TEM (HRTEM) and STEM (including EDS) images were obtained using a JEOL 2100F transmission electron microscope. Zeta potential changes and DLS-measured diameters were measured using a Zeta PALS Submicron Granulometer (Brookhaven Instruments Corp., Holtsville, NY, USA). Magnetic properties were investigated using a vibrating sample magnetometer (VSM, Lake Shore 7307). UV-Vis-NIR spectra were acquired using a PerkinElmer Lambda 750 UV-Vis spectrophotometer (Waltham, MA, USA). The magnetic hyperthermia effect was characterized using a copper-constantan thermocouple (magnetic field frequency, 200 kHz; field amplitude, 38 KA/m). NPTT was induced by a near-infrared continuous laser at 808 nm (GSCLS-05-700W; Daheng Group, Inc., Beijing, China). The temperature induced by photo-magnetic hyperthermia was recorded using an infrared thermal imaging camera (Fluke IT200, Everett, WA, USA).

**Cellular Experiments:** MCF-7 human breast cancer cells and L929 murine fibroblast cells were purchased from Peking Union Medical College (PUMC). All cell culture reagents were purchased from HyClone (Logan, UT, USA). *In vitro* cytotoxicity was measured using a standard Cell Counting Kit (CCK8) (Sigma–Aldrich, St. Louis, MO, USA) assay. MCF-7 and L929 cells were seeded in 96-well cell culture plates at 1 × 10^4/well and were then incubated for 24 h at 37 °C under 5% CO₂. After incubation with various concentrations of free MNP@PES-Cy7/2-DG for 24 h and 48 h, the standard CCK8 assay was performed to determine cell viability relative to that of untreated cells. Confocal laser scanning microscopy (CLSM) images of MCF-7 cells, mouse L929 fibroblast cells and mouse bone marrow derived macrophage RAW 264.7, after incubation with NPs for 4 h, were obtained using the Leica Microsystem (TCS SP5, Wetzlar, Germany). Cell nuclei were stained with DAPI and cytoskeletons were stained with RhB.

For three-mode hyperthermia, samples were subjected to NMH with AMF for 10 min, NPTT alone, 808 Laser (0.75 W/cm²) for 10 min; for dual-mode, samples were treated with AMF plus the laser for 10 min. The suspension of NP-loaded cells was placed inside the coil in 2-mL Eppendorf tubes to measure the temperature *in vitro*. The temperature increase was recorded using an infrared thermal imaging camera in real time. After incubation with NPs for 4 h and different treatments, cells were incubated for an additional 24 h at 37 °C. Then, a standard CCK8 assay was performed as described previously. Live/dead assays were used to investigate the effects of different hyperthermia
therapies. After applying different hyperthermia treatments, the culture medium was removed and PBS containing 2.0 μM Calcein-AM (Sigma-Aldrich) and 1.0 μM Propidium Iodide (PI) (Sigma-Aldrich) was added. Microscopic images of cells were then obtained using a Leica microscope under laser excitation at 475 nm and 542 nm. Cells were stained with PI (Sigma-Aldrich) and Annexin V-FITC (Sigma-Aldrich) for 15 min. The stained cells were examined using a flow cytometer (BD FACSCalibur, Franklin Lakes, NJ, USA), and data were analyzed using FlowJo.

Animals and Tumor Model: All experiments involving animals were performed in accordance with the guidelines of the IACUC of Tsinghua University, Beijing, China. BABL/c nude mice (4–5 weeks old) with an average weight of 18 g were provided by Weitong Lihua Experimental Animal Technology Co. Ltd., Beijing, China. Mice were injected with MCF-7 cells (0.2 mL in H-DMEM culture medium without FBS, ≈5 × 10^6) subcutaneously at the right rear flank. The mice were used when their tumor volumes approached 80–100 mm^3.

Blood Circulation: In the pharmacokinetic analysis, blood circulation was measured by drawing ~10 μL of blood from the tail vein of MCF-7 tumor bearing mice post-injection of MNP@PEDOT-Cy7/2-DG. Each blood sample was dissolved in 1ml of the lysis buffer ((1% SDS, 1% Triton X-100, 40 mM Tris acetate). And the concentration of the NPs in the blood was determined from the fluorescence spectrum acquired on a fluorometer with the excitation and emission peaks at~747nm and~774 nm. A series of NPs dilutions were performed to obtain a standard calibration curve. Blank blood sample without NPs was measured to determine the blood auto-fluorescence level, which was subtracted from the fluorescence intensities of injected NPs during the concentration calculation.

Biodistribution Measurement: For the quantitative biodistribution analysis, MCF-7 breast-tumor-bearing mice were sacrificed at 12, 24 and 48 h post-injection of MNP@PEDOT-Cy7/2-DG. The major organs and tumor were weighed and homogenized in the lysis buffer (the same as the above). Clear homogeneous tissue solutions were diluted about 100 times to avoid light scattering and self-quenching during fluorescence measurement. The samples were measured four times following the above methods to ensure reproducibility and accuracy.

Multimodality Imaging: For in vivo fluorescence molecular imaging, BALB/c mice were intravenously injected with MNP@PES-Cy7/2-DG (0.15 mL of a 0.5 mg/mL solution for each mouse) and fluorescent signals were detected at various time points, such as before treatment and at 0.5 h, 1 h, 4 h, 8 h, 12 h, 24 h, 36 h, and 48 h. For in vivo MR imaging and PAI, mice received i.v. injection with the same dose of MNP@PES-Cy7/2-DG and images were obtained before injection and 24 h post-injection using a clinical 3T MRI scanner (Philips) or a multispectral photoacoustic tomography system.
In vivo hyperthermia treatment: For three-mode hyperthermia, 5 groups (6 mice per group) were treated as follows: (1) saline (0.2 mL per mouse); (2) saline with laser plus AMF; (3) MNP@PES-Cy7/2-DG (0.2 mL of 2.0 mg/mL per mouse) with a laser (808 nm, 0.75 W/cm², 10 min); (4) MNP@PES-Cy7/2-DG with AMF (200 kHz, 38 KA/m, 10 min); (5) MNP@PES-Cy7/2-DG with a laser plus AMF (10 min), at 24 h after injection. The temperature was monitored in vivo using an infrared thermal imaging system (Fluke, IT200). Tumor sizes and body weights were measured every 2 days during the treatment period.

Histological analysis: Major organs, including the liver, spleen, kidney, heart, and lung, were obtained from mice, fixed in 10% neutral-buffered formalin, processed routinely in paraffin, sectioned at 5 μm, stained with Prussian blue or hematoxylin and eosin (H&E), and examined using an optical microscope (OLYMPUS IX81-ZDC, CCD: DP80).

Statistical analysis: All the experiments were carried out at least three times (n = 3–6). And all data were expressed as means ± standard deviation (SD) values. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by post hoc comparisons with least significant difference (LSD) method using SPSS 22.0 software to evaluate differences between two groups. Values of p < 0.05, 0.01 and 0.005 were considered statistically significant, high significant and extreme high significant respectively.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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References


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A novel multifunctional theranostic nanoplatform (MNP@PES-Cy7/2-DG) has been developed and applied for combined photo-magnetic hyperthermia therapy under intravenous administration which was simultaneously guided by multimodality molecular imaging. Under the guidance of PAI/MAI/FMI tri-modality imaging, tumors could be completely eliminated under intracellular inside-out hyperthermia with additive therapeutic effect due to precise hyperthermia.
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