Nuclear-Targeted Multifunctional Magnetic Nanoparticles for Photothermal Therapy

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The pursuit of multifunctional, innovative, more efficient, and safer cancer treatment has gained increasing interest in the research of preclinical nanoparticle-mediated photothermal therapy (PTT). Cell nucleus is recognized as the ideal target for cancer treatment because it plays a central role in genetic information and the transcription machinery reside. In this work, an efficient nuclear-targeted PTT strategy is proposed using transferrin and TAT peptide (TAT: YGRKKRRQRRR) conjugated monodisperse magnetic nanoparticles, which can be readily functionalized and stabilized for potential diagnostic and therapeutic applications. The monodisperse magnetic nanoparticles exhibit high photothermal conversion efficiency ($\approx$37%) and considerable photothermal stability. They also show a high magnetization value and transverse relaxivity ($207.1\,\text{m}\text{T}s^{-1}$), which could be applied for magnetic resonance imaging. The monodisperse magnetic nanoparticles conjugated with TAT peptides can efficiently target the nucleus and achieve the imaging-guided function, efficient cancer cells killing ability. Therefore, this work may present a practicable strategy to develop subcellular organelle targeted PTT agents for simultaneous cancer targeting, imaging, and therapy.

1. Introduction

Photothermal therapy (PTT), which utilizes heat generated from photothermal agents under near-infrared (NIR) light irradiation to burn cancer, as a minimally invasive local cancer treatment has attracted increasing attention in the past decade.[1,2] Compared with surgical management, chemotherapy, and radiotherapy, PTT is highly efficient in tumor ablation and exhibits minimal damage to normal tissues.[3] To date, a broad range of materials, such as gold nanoarchitectures,[4,5] carbon nanomaterials,[6,7] and organic polymers (e.g., polydopamine,[8] polypyrrole[9]) have been investigated as PTT agents. Majority of these studies have focused on enhancing the photothermal efficacy of the nanoparticles by tuning their particle size,[10,11] morphology,[12] surface charge,[13] and coating compositions (e.g., polydopamine,[14] or polypyrrole[15]). To achieve a high photothermal ablation effect, the NIR laser irradiation is typically applied in the tumor area after the photothermal agents having been accumulated in the tumor tissues. For instance, Tan and co-workers have reported that an aptamer switch probe linking to the surface of gold nanorods was used to target cancer cells for PTT and photodynamic therapy (PDT), providing high specificity and therapeutic efficiency.[16] Since many diseases are caused by alterations at molecular or nanoscale levels. Therapeutic drugs are expected to deliver at organelle or subcellular organelle level to achieve maximum therapeutic effect.[17] It is reported that selectively delivering PTT nanoparticles to subcellular organelle (mitochondria) can realize higher photothermal efficacy.[18,19] Shi and co-workers reported a small-molecule-based cancer theranostic agents integrated both cancer PTT and PDT treatment by targeting mitochondria, thus significantly increasing the phototherapeutic efficacy.[20] As the cell nucleus is the cellular “heart,” it is the ultimate targeting destination, where plentiful therapeutic agents efficiently work and where the transcription machinery reside as well.[21] Considering the extraordinary importance in nuclear-targeted drug delivery, various functional nanoparticles (e.g., gold nanoparticles,[22–24] silver nanoparticles,[25] quantum dots,[26] etc.) have been showed to be of potential use in targeting nuclei through conjugation of nuclear localization signal (NLS) to the surface of the nanoparticles. TAT peptide (TAT: YGRKKRRQRRR) (a kind of NLS) proves to be an efficient molecule for translocating nanoparticles into cell nuclei via binding imported receptors importin $\alpha$ and $\beta$ (karyopherin), subsequently targeting the nuclear pore complexes (NPCs) and finally entering their nuclei.[27,28] Nucleus, which is a desired target for diseases, is the central governor of cell reproduction, metabolism, and cell cycle. The nuclear targeting therapy strategy has been applied in drug...
delivery or PDT.\textsuperscript{29} For instance, Shi and co-workers have proposed an effective strategy for combining TAT peptide with mesoporous silica nanoparticles to target the nucleus and then deliver anticancer drugs or photosensitizers, resulting in a significantly enhanced anticancer activity of the drug or PDT (photosensitizers).\textsuperscript{30,31}

Among the various types of PTT agents, magnetic particles\textsuperscript{32} such as iron oxide nanoparticles (IONPs) are excellent candidates for photothermal therapy anticancer therapy, because they are nontoxic, biocompatible, and compatible with magnetic resonance imaging (MRI) technology.\textsuperscript{33–35} For example, gold nanorods lack photosensitivity due to a “melting” phenomenon resulting from point and planar defects.\textsuperscript{36} Gold nanoshells are too large to meet the requirement of enhanced permeability and retention effect.\textsuperscript{37} Furthermore, the application of carbon nanotubes is also limited by potential long-term toxicity and many toxic responses such as oxidative stress and pulmonary inflammation.\textsuperscript{39,40} However, Li and co-workers reported a convenient method of thermal decomposition for making highly crystallized iron oxide nanoparticles with the controlled size from 5 to 40 nm.\textsuperscript{41} Magnetic particles also have great potential for clinical applications due to their biocompatibility, biodegradability, and well-characterized pharmacokinetics.\textsuperscript{42} Therefore, magnetic nanoparticles possess with the following advantages: small size, easy functionalization, biocompatible, biodegradability, photosensitivity, and dual-modality. However, the nuclear-targeted PTT cancer treatment with magnetic nanoparticles has not been reported to our best knowledge.

Herein, we report an efficient strategy to improve PTT treatment by selectively delivering IONPs to cell nuclei. The IONPs were functionalized with TAT for efficient nuclear targeting and fluorescein dyes (i.e., fluorescein isothiocyanate, FITC, or cyanine7, Cy7) for fluorescence-based real-time tracking of the particles uptake. Quantitative analysis confirmed that the multifunctional IONPs have a significantly higher accumulation in the nuclei (122 μg Fe per mg protein), which was 45-fold higher than those without TAT modification. Furthermore, the multifunctional nanoparticles are able simultaneously used as agents for NIR fluorescence imaging and MRI. After in vivo administration, the composite nanoparticles are able to ablate tumor xenograft effectively with NIR laser exposure and are hopeful for cancer treatment.

2. Results and Discussion

As we all know, a small enough particle size is an important prerequisite to ensure that the nanoparticles can step across NPCs. Therefore, three small size nanoparticles (20, 11, and 5 nm) are synthesized to realize the nuclear internalization by cancer cells. Typically, highly monodisperse oleic acid (OA)-stabilized IONPs with uniform and tunable particle size (OA-IONP-20, OA-IONP-11, and OA-IONP-5) were prepared following the protocol proposed by Hyeon and co-workers with a slight modification.\textsuperscript{43} As illustrated in Scheme 1, a multifaceted targeting nanomedicine is functionalized by covalently conjugating transferrin (Tf)\textsuperscript{44} and TAT peptides onto the highly monodispersed IONPs. First, we use a facile, high efficiency and low-cost method to convert OAA-stabilized IONPs to 3,4-dihydroxyphenylalanine (DHCA)-stabilized IONPs using tetrahydrofuran (THF), NaOH, and DHCA without any complicated organic synthesis according to the literature.\textsuperscript{45} DHCA-stabilized IONPs neutralized with sodium hydroxide showed excellent water dispersible and stability in biological environments (denoted as IONP-20, IONP-11, and IONP-5). DHCA anions not only offer IONPs excellent water dispersibility but also provide a platform for further surface functionalization via the carboxyl group to form an amide linkage with amine-containing molecules. Second, the carboxyl group in the synthesized IONPs react with amino groups in TAT and NH₂-PEG-Mal, forming an amide bond by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and n-hydroxysuccinimide (EDC/NHS) (denoted as IONP-TAT/PEG-Mal). Third, Cy7 as a near-infrared fluorescence (NIRF) dye is immobilized onto the surface of IONPs through the reaction between the carboxyl and amino groups, denoted as IONP-TAT/Cy7/PEG-Mal. Then, Tf is treated with 2-iminohydroxyl chloride to obtain the thiolated transferrin (Tf-SH). Lastly, IONP-TAT/Cy7/PEG-Mal-Tf (IONP-TAT/Cy7-Tf) is obtained after Tf-SH was anchored onto the end of the PEG-Mal chains through the reaction between a sulfhydryl group and a maleimide end group.

IONP-20, IONP-11, and IONP-5 nanocrystals could be observed from the transmission electron microscope (TEM) images (Figure 1a,b; Figure S1, Supporting Information). All the nanocrystals are highly monodisperse in particle-size distribution. Well-resolved lattice fringes can be observed from the high-resolution transmission electron microscope (HRTEM) images, corresponding to d spacing values of 0.28 and 0.21 nm (IONP-20; Figure 1a, inset), which are close to the (100) planes of a cubic spinel structure of magnetite. While the d spacing values of IONP-11 are 0.25 and 0.41 nm (Figure 1b, inset), which are close to the (311) planes of a cubic spinel structure of magnetite. The fast Fourier transform (FFT) patterns confirmed that the obtained IONP-20 and IONP-11 are single crystal line (Figure 1a,b, inset). Moreover, scanning transmission electron microscope–energy dispersive X-ray spectroscopy (STEM-EDX)}
IONPs particles dispersions acquired by MR scanner exhibit increasingly darker/brighter contrast as an increased Fe concentration (Figure 1e). The $r_1$ relaxivity value of IONP-5 was calculated to be 5.42 mM$^{-1}$ s$^{-1}$ (Figure 1f), demonstrating that IONP-5 could be efficient $T_1$ contrast agent. The high $r_1$ relaxivity of IONP-5 can be attributed to the large number of surface Fe$^{3+}$ ions with five unpaired valence electrons.[47] Compared to IONP-5, IONP-11 and IONP-20 exhibited stronger $T_2$ MR contrast effects.[48,49] The relaxivity coefficient ($r_2$), which was a direct indication of contrast enhancement effects, was 67.1 mM$^{-1}$ s$^{-1}$ for IONP-5 and gradually increased to 207.1 mM$^{-1}$ s$^{-1}$, and to 267.9 mM$^{-1}$ s$^{-1}$ for IONP-20 and IONP-11 (Figure 1g). It is noteworthy that the $r_2$ of IONP-20 is higher than that of commercial MRI contrast agents (Feridex, 93 mM$^{-1}$ s$^{-1}$).[50]

To study the potential use of the IONPs as PTT agents, we next investigated the photothermal properties of IONPs induced by the NIR laser irradiation. The absorbance in the NIR region is one of the key factors that determine the photothermal conversion capability of a PTT agent. To explore how effectively the nanomaterials could absorb light, we summarized their vis–NIR spectra (at an equivalent concentration of 100 µg mL$^{-1}$) in Figure 2a. Compared with IONP-5, IONP-20 presented a remarkable increase in NIR absorption of 808 nm at an equivalent mass concentration, indicating that the NIR absorption at 808 nm could be improved by increasing the size of the IONPs. When IONPs were exposed to the laser, we monitored the temperature change of nanoparticle suspensions under continuous NIR laser irradiation (808 nm, 3 W cm$^{-2}$, 500 s, spot size 6 × 7 mm$^2$). Owing to the NIR absorbance, an aqueous dispersion of IONP-20 could be rapidly heated up to ~63 °C after being irradiated (Figure 2b). In contrast, the temperature was raised to 52 °C and 44 °C of IONP-11 and IONP-5. IONPs also exhibited concentration-dependent photothermal heating effect (Figure 2c). We also monitored the photothermal effects of IONP-20, IONP-20-TAT, IONP-20-Tf, and IONP-20-TAT-Tf aqueous dispersions (total 100 µg mL$^{-1}$) and equivalent amount of Fe$_3$O$_4$ of 100 µg mL$^{-1}$ (iron element was measured by inductively coupled plasma atomic emission spectrometry). The temperature was measured using a NIR camera (VarioCAM HR, InfraTec, Germany). Compared to IONP-20, IONP-20-TAT-Tf showed a lower photothermal effect at an equivalent concentration (100 µg mL$^{-1}$), due to the existence of TAT and Tf (Figure S2a, Supporting Information). In other words, TAT and Tf made little contribution to photothermal effect. The photothermal heating ability of IONP-20-Tf was about 50% lower than that of IONP-20, and the IONP-20-TAT-Tf was 40% lower than that of IONP-20-TAT.
conversion efficiency (37%) than that of gold nanorods (22%)\[52\] and gold nanoshells (13%)\[53\] eventually resulting in their excellent photothermal effects (Table S2, Supporting Information). The result revealed that IONP-20 had a little better photothermal effect compared with PDA at the same condition (Figure S4, Supporting Information).

To further illustrate the photothermal performance of the IONPs, we did further investigation. The previous structural characterizations and quantitative identification of different sized iron oxide nanoparticles using X-ray absorption spectroscopy and X-ray magnetic circular dichroism spectroscopy showed that iron oxide nanocrystals can be expressed as (γ-Fe₂O₃)ₓ-α(Fe₃O₄)₉ according to the literature.\[43\] The estimations of x are 0.20, 0.68, and 1.00 for the 5, 12, and 22 nm nanocrystals, respectively. Therefore, γ-Fe₃O₄ is the dominant phase of the 5 nm iron oxide nanocrystals, whereas the proportion of the Fe₃O₄ component gradually rises as particle size increases. It is reported that Fe₂O₃ particles have a strong absorption at 808 nm while Fe₃O₄ particles have no NIR absorption at 808 nm.\[54\] Although IONP-20 exhibited a photothermal conversion efficiency lower than that of IONP-5, it showed a higher absorbance value at 808 nm (Figure 2a), eventually resulting in better photothermal effects than those of IONP-5. These results revealed that the superior photothermal effect of IONP-20 was attributed to the NIR absorption rather than the photothermal conversion efficiency. Note that photothermal effect and size effect of across NPCs,\[22\] 20 nm of IONP-TAT-Tf (denoted as IONP-20-TAT-Tf) was chosen for the following cell and animal experiment as the ideal nanoparticle.

Cytotoxicity of IONPs was investigated through the standard 3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay on normal cells (HEK 293T). Due to the good biocompatibility of IONPs, HEK 293T cells treated with nanoparticles display over 90% survival rate, which indicated no obvious cytotoxicity (Figure S5, Supporting Information). To investigate cellular uptake efficiency of IONP-20-TAT-Tf over the time, the particles were coincubated with human lung carcinoma (A549) cells and then observed using confocal laser scanning microscopy (CLSM). To visualize the location of multifunctional nanoparticles in subcellular level, FITC was attached covalently onto the TAT (FITC-C6-TAT). The vis-NIR spectrum of the IONP-FITC-C6-TAT shows the characteristic absorption bands of FITC at the N-termini of the TAT peptides at 490 nm, which indicates the successful conjugations of TAT peptides on IONP (Figure S6, Supporting Information). The cellular uptake and subsequent localization of IONP-20-TAT-Tf for 1, 2, and 4 h incubations were shown in Figure 3a. It could be seen from the figure that there were very few nanoparticles in the nuclei after 1 h. However, IONP-20-TAT-Tf could be clearly observed in both the cytoplasm and the nuclei of IONP-20, IONP-20-TAT, IONP-20-Tf, and IONP-20-TAT-Tf are similar at equivalent amount of Fe₃O₄, which indicated that functionalization (Tf and TAT) just has little photothermal effect of IONP (Figure S2b, Supporting Information). Furthermore, the photothermal stability of IONPs was confirmed (Figure 2d). Additionally, the photothermal effect of IONPs is only slightly decreased over a period of 16 weeks. As shown in images in Figure 2d (inset), the color and colloidal stability of the samples were largely remained even after 16 weeks of storage in dispersion.

The photothermal conversion efficiency (η) is another key parameter that determines the conversion efficacy of a photothermal agent. Therefore, we compared the photothermal conversion capability of different size of IONPs with some other photothermal conversion agents (Table S1, Supporting Information). We calculated the photothermal conversion efficiency based on the previous report\[51\] and detailed calculation is given as equation in the Supporting Information. The η (Figure S3, Supporting Information) of IONP-5, IONP-11, and IONP-20 are around 43%, 32%, and 37%, respectively (Table S1 in the Supporting Information). Even though the measured η value of IONP-5 is the highest in IONPs, the low NIR absorption at 808 nm of IONP-5 lead to the weaker photothermal effect. Although IONP-20 exhibited a lower absorption coefficient (1.22 × 10⁴) than that of polypyrrole (1.75 × 10⁴) and gold nanorods (4.28 × 10⁴), it showed a higher photothermal efficiency based on the previous report,\[51\] and detailed calculations. We calculated the photothermal conversion efficiency (η) is another key parameter that determines the conversion efficacy of a photothermal agent. Therefore, we compared the photothermal conversion capability of different size of IONPs with some other photothermal conversion agents (Table S1, Supporting Information). We calculated the photothermal conversion efficiency based on the previous report\[51\] and detailed calculation is given as equation in the Supporting Information. The η (Figure S3, Supporting Information) of IONP-5, IONP-11, and IONP-20 are around 43%, 32%, and 37%, respectively (Table S1 in the Supporting Information). Even though the measured η value of IONP-5 is the highest in IONPs, the low NIR absorption at 808 nm of IONP-5 lead to the weaker photothermal effect. Although IONP-20 exhibited a lower absorption coefficient (1.22 × 10⁴) than that of polypyrrole (1.75 × 10⁴) and gold nanorods (4.28 × 10⁴), it showed a higher photothermal efficiency based on the previous report,\[51\] and detailed calculation is given as equation in the Supporting Information. The efficiency of IONP-20, IONP-20-TAT, IONP-20-Tf, and IONP-20-TAT-Tf are similar at equivalent amount of Fe₃O₄, which indicated that functionalization (Tf and TAT) just has little photothermal effect of IONP (Figure S2b, Supporting Information). Furthermore, the photothermal stability of IONPs was confirmed (Figure 2d). Additionally, the photothermal effect of IONPs is only slightly decreased over a period of 16 weeks. As shown in images in Figure 2d (inset), the color and colloidal stability of the samples were largely remained even after 16 weeks of storage in dispersion.

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of A549 cells after incubation for 2 and 4 h, as demonstrated by the green fluorescence from FITC lighting up the nuclei. It was concluded that the IONP-20-TAT-Tf can penetrate into nuclei in 4 h. In addition, flow cytometry graphs (Figure S7, Supporting Information) further confirmed the effective intra-nuclear localization of IONP-20-TAT-Tf in 4 h. Moreover, the in situ monitoring and visualizing of the processes of IONP-20-TAT-Tf delivery/diffusion clearly showed the enhanced cellular and nuclear uptake of IONP-20-TAT-Tf (Movie S1 in the Supporting Information). Furthermore, the biotransmission electron microscopy (Bio-TEM) images after incubation with these particles strongly supported our claims. We can clearly observe IONP-20-TAT-Tf inside the nuclei (Figure 3c) and IONP-20 mostly outside the nuclei (Figure 3b). Our results are comparable to the conclusion drawn by Panté and Kann that nanoparticles with a diameter close to 39 nm could be translocated into the cell nucleus. Since TAT peptide could assist the importing of 90 nm beads into the nuclei of digitonin-permeabilized cells. The nuclear uptakes of IONP-20 and IONP-20-TAT-Tf were also confirmed by nuclear iron element quantification via extraction of nuclei from the cells. Quantitative analysis confirmed that IONP-20-TAT-Tf provided a significantly higher accumulation in the nuclei (122 µg Fe per mg protein), which was 45-fold higher than IONP-20 (Figure 3d). It is known that the free TAT peptide does not facilitate the nuclear location.

To verify the intracellular localization of IONP-20 and IONP-20-TAT-Tf, we make a further study of intracellular localization experiment by Bio-TEM at different incubation time (4 and 12 h). From the Bio-TEM (Figure S8i–iii, Supporting Information), IONP-20 was indeed not free in the cytoplasm but accumulated in endosome/lysosome with 4 h incubation. When the incubation time reached to 12 h, it can be observed that the IONP-20 was still entrapped in the endosomes or lysosome (Figure S8iv–vi, Supporting Information). Otherwise, IONP-20-TAT-Tf can avoid endo/lysosomal

Figure 3. In vitro cell experiment. a) CLSM images of A549 cells incubated with IONP-20-TAT-Tf. For each panel, the images from left to right show cell nucleus stained by FITC fluorescence from FITC-C6-TAT in cells (green), DAPI (blue: DAPI = 4′,6-diamidino-2-phenylindole), and the overlays of the former two images. Bio-TEM images of A549 cells incubated with b) IONP-20 and c) IONP-20-TAT-Tf for 12 h. C = cytoplasm, N = nucleoplasm. d) Cellular and nuclear uptake amounts of IONP-20 and IONP-20-TAT-Tf by A549 cells.
entrapment, escape the endosome and then further translocate across the NPC into the cell nucleus (Figure 4c). When the incubation time reached to 12 h, it can be observed that IONP-20-TAT-Tf largely accumulated in the nucleus (Figure 3c).

In order to examine the processes of intracellular trafficking, a fluid-phase endosomal marker Alexa Fluor 594-labeled Dextran (Dextran, Alexa Fluor 594; $M_w$: 10 kDa), was used to monitor endocytosis pathway. The lysosomes of A549 cells are stained with Lysotracker (blue). As shown in Figure 4a, IONP-20-TAT-Tf, with targeting ligand on the surface, are expected to enter into cancer cells through Tf receptor-mediated endocytosis, escape endosomal/lysosomal pathways (Figure 4b),[58] and further target to nucleus due to the TAT peptides.[59] After A549 cells were incubated with IONP-20-TAT-Tf for 1 h, the vesicular structures are able be visualized clearly in the microscope image (Figure S9, Supporting Information), indicating the Tf-mediated endocytosis.[60] Shortly after internalization (1 h), only slight colocalization was detected with IONP-20-TAT-Tf (Figure 4b), whereas extensive colocalization endosome maker dextran and lysosome maker was observed. In addition, the endosomal marker Dextran showed clear colocalization with IONP-20-TAT-Tf at 2 h (Figure 4b; Figure S10, Supporting Information). Further experiments demonstrated the colocalization of the IONP-20-TAT-Tf and Alexa Fluor 594-labeled Dextran at the nuclear periphery at 2 h, implying that most of the IONP-20-TAT-Tf was unable to escape from the intracellular endosome. When endosome released IONP-20-TAT-Tf, however, a major cell nuclear accumulation was observed at 4 h (Figures 3a and 4b). In addition, Figure 4c (ii) also demonstrated that IONP-20-TAT-Tf can avoid endo/lysosomal entrapment and escape the endosome at 2–4 h post-transduction. The escaped IONP-20-TAT-Tf can accumulate near the nucleus and translocate across the nuclear pore complex into the cell nucleus (Figure 4c (iii)). When the incubation time reached to 12 h, it can be observed that IONP-20-TAT-Tf largely accumulated in the nucleus (Figure 3c).

Then calcein acetoxymethyl ester (calcein-AM) was used to stain live cells (green) whereas propidium iodide (PI) was used to stain dead cells (red) under confocal fluorescence imaging (Figure 5a).[61] In the CLSM pictures, cells incubated with IONP-20-TAT-Tf under laser treatment showed strong red fluorescence, implying that severe cell death were
Figure 5. a) CLSM images of IONP-20-Tf and IONP-20-TAT-Tf incubated A549 cancer cells after irradiation by the 808 nm laser at 3 W cm\(^{-2}\) for 5 min. The cells were costained by calcein-AM and propidium iodide before imaging. b) Flow cytometry graphs of A549 cells treated by IONP-20, IONP-20-Tf, IONP-20-TAT, and IONP-20-TAT-Tf with a concentration of 50 \(\mu\)g mL\(^{-1}\) (NIR laser \(\lambda = 808\) nm, 1.5 W cm\(^{-2}\), 300 s).
induced by IONP-20-TAT-Tf via photothermal effect. In contrast, cells without laser treatment, gave out bright green fluorescence from calcein-AM staining, while remaining dark in PI channel, indicating high cell viabilities. The mechanism was that Tf units on the surface of IONP-20-TAT-Tf could improve the internalization efficiency of the nanoparticles through specific receptor-mediated endocytosis. Cells exhibited significantly higher PI signal in IONP-20-TAT-TF treated group than that in IONP-20-TF treated group, indicating that the decreased cell viability is indeed induced by TAT. Therefore, TAT further improves photothermal efficacy because of the nuclear targeting. To clarify the cell death mode after photothermal treatment, an annexin V-FITC/PI method was conducted by flow cytometry. Figure 5b exhibited the flow cytometry graphs of the cells by IONP-20, IONP-20-TF, IONP-20-TAT, and IONP-20-TAT-TF with laser irradiation. The apoptosis rate of cells of IONP-20-TAT-Tf under laser irradiation reached 54.1%, while that of IONP-20-TF was only 16.0%. The flow cytometry data revealed that cells treated by IONP-20-TAT-TF displayed irreversible damage upon photothermal treatment and give rise to the apoptotic process. That is, the mechanism of cell death was largely triggered by cell apoptosis. Taken together, IONP-20-TAT-TF can be efficiently delivered into nuclei of cancer cells via nuclear-targeted strategy, there after causing superior photothermal efficacy. We also evaluated the relative viabilities of A549 cells with different laser power, duration time and concentration of the PTT agents (Figure S11, Supporting Information). In the case of reducing laser power, we can increase the concentration of the PTT agents and the time of illumination to achieve the equal photothermal effects.

The nuclear-targeted strategy may prevent IONPs-TAT-TF from degradation in endosomes (pH 5.5–6.0) or lysosomes (pH 4.5–5.0) and then lead to a better stability. Beside, heat-shock proteins (HSPs) are families of highly conserved proteins that are induced in cells by a large variety of stresses, including heat stress, heavy metals, hypoxia, and acidosis. Because expression of the HSP70 protects cells from heat-induced apoptosis and the cells acquire thermosterance, their levels of intracellular expression increase in response to protein-denaturing stressors, such as temperature change, as an evolutionarily conserved response to restore the normal protein-folding environment and to enhance cell survival. The transcription machinery impairment of the nuclear might down-regulate the expression of the HSPs. The cells of low thermostolerance will undergo heat-induced apoptosis.

Utilizing magnetic properties of IONP-20-TAT-TF, in vivo MR imaging on tumor-bearing mice has been studied by a 7 T MR scanner. MRI is one of the most powerful medical diagnosis tools. MRI can provide images with excellent anatomical details contrast and functional information in real-time monitoring manner. To investigate whether the IONP-20-TAT-TF would guide the tumor specific enrichment of the nanoparticles, bab/c mice bearing A549 tumors were intravenously (i.v.) injected with IONP-20-TAT-TF (200 µL, 0.5 mg mL⁻¹). Those mice were then imaged at various time intervals. Notably, compared to the 0 h, the MR images of targeted tumors demonstrated dramatic darkening effect (white dashed circles) at the point of 8–12 h postinjection, indicating the high accumulation of IONP-20-TAT-TF nanoparticles in tumor (Figure 6a). To confirm the MR results, in vivo fluorescence imaging (Figure 6b) was tested at different time points of the Cy7 modified IONP-20-TAT-TF (denoted as IONP-20-TAT-Cy7-TF). IONP-20-TAT-Cy7-TF (a dosage of 5 mg kg⁻¹) was injected i.v. and the NIRF imagings were acquired at various time postinjection. It was found that IONP-20-TAT-Cy7-TF tended to be enriched in the tumor area, in which the Cy7 fluorescent signal showed an obvious increase over time after injection. The maximum NIR signal at 8 h postinjection could be observed in the tumor area, suggesting that IONP-20-TAT-Cy7-TF could accumulate in tumor.

The combination of the two modes of in vivo imaging endowed IONP-20-TAT-Cy7-TF with great prospect for imaging guided photothermal therapy. Both MRI and fluorescence imaging results suggest the significant signals at 8 h after the injection of the particles, while it was considered that IONP-20-TAT-TF need another 4 h penetrate into nuclei (Figure 3a). Taken together, these results indicated that laser intervention could have optimized effect when performed about 12 h after the injection. We can conclude that the two processes above are of great importance and can provide valuable information for better therapeutic planning.

Encouraged by the excellent efficacy achieved and imaging test, oncotherapy experiments in vivo were then conducted on lung cancer tumor model. Five groups were included: (1) PBS (phosphate buffer saline) + laser, (2) IONP-20 + laser, (3) Tat modified IONP-20 (denoted as IONP-20-TAT) + laser, (4) Tf modified IONP-20 (denoted as IONP-20-TF) + laser, (5) IONP-20-TAT-TF + laser (n = 5 per group). Five groups of A549-bearing mice were i.v. injected with 0.2 mL of PBS or nanoparticle suspensions (0.5 mg mL⁻¹) to the mice with diameters of 5 mm. 12 h postinjection, the mice were exposed to the 808 nm NIR laser at the density of 3 W cm⁻² for 300 s. The temperature of the mice was monitored using the thermal imaging real time (Figure 6c). We observed that the temperature rise of PBS group was neglectable. For IONP-20-TF and IONP-20-TAT-TF, the tumor temperature increased more rapidly within 300 s than the temperature of tumor containing IONP-20 or IONP-20-TAT. To evaluate the anticancer efficacy of IONPs, tumor growth rates (Figure 7b) were recorded. The results showed the tumors in group PBS and group IONP-20 grow rapidly, due to the inefficient enrichment of nanoparticles in tumor region. The IONP-20-TF groups exerted a slower growth rate, suggesting the better enrichment of nanoparticles in tumor region. The IONP-20-TAT-TF group was exerted significantly slow growth rate, indicating the successful therapeutic effect of nuclear-targeted PTT. The statistically significant difference was observed in group IONP-20-TAT-TF and other groups. At the 14th day, tumors were excised and weighted (Figure 7c). The average weights of tumors for group PBS, IONP-20, IONP-20-TAT, IONP-20-TF, and IONP-20-TAT-TF were showed in Figure 7c, respectively. The calculated tumor inhibition rates are 29.84% for IONP-20 treated group, 47.91% for IONP-20-TAT treated group, 70.60% for IONP-20-TF treated group, and 90.85% for IONP-20-TAT-TF treated group. Altogether, the in vivo PTT results consistently imply IONP-20-TAT-TF own excellent therapeutic effect in the phototherapy of cancer.

To confirm the safety of using IONPs in PTT, we performed histology analysis via standard histological techniques with
hematoxylin and eosin (H&E) staining. Mice i.v. injected with IONP-20-TAT-Tf were sacrificed 14 d after PTT treatment, with major organs collected and sliced for histology analysis. Neither noticeable tissue damage nor adverse effects to the main organs could be observed (Figure 7d; Figure S12, Supporting Information), preliminarily proving that IONP-20-TAT-Tf was not noticeably toxic at the tested dose to animals. Neither obvious body weight loss (Figure 7a) nor abnormal behavior was observed for IONP-20-TAT-Tf injected mice. To sum up, these observations suggest that the treatment of IONPs, besides effective in eliminating tumor via PTT, poses no obvious signals of toxic side effects in mice.

3. Conclusion

In summary, we have developed readily functionalized and stabilized monodisperse magnetic nanoparticles simultaneously with cancer cell nuclear targeting, MR/NIR imaging, and synchronous PTT effects. The IONP-20-TAT-Tf successfully realizes the phototherapy upon NIR laser irradiation and greatly enhances the therapeutic effect by nuclear targeting. Additionally, the MR/NIR imaging makes tumors with the margins clearly visualized, which is greatly helpful for imaging-guided phototherapy and real-time treatment monitoring. Moreover, it exhibits good PTT effects by targeting nucleus, thus leading to a significant enhancement in the photothermal therapy. In vivo experiments confirm the MR/NIR imaging functions and remarkable tumor inhibition effect of IONP-20-TAT-Tf, while having little toxicity. Therefore, NIR light induced and nuclear targeting nanoparticles are expected to have wide applications in the cancer therapy.

4. Experimental Section

Materials: EDC, NHS, and Tf were obtained from Sigma-Aldrich. TAT (YGRKKRRQRRR) and FITC-C6-TAT were purchased from Chinese Peptide Company. Amino Polyethylene Glycol Maleimide (NH$_2$-PEG-Mal, $M_w = 2000$ Da) were purchased from Beijing Jenkem Technology Company (Beijing, China). 2-[4-(2-hydroxyethyl)-1-piperazinyl]...
ethanesulfonic acid (HEPES), 2-iminothiolane hydrochloride and 5,5-dithiobis (2-nitrobenzoic acid) were obtained from Sigma-Aldrich (USA). NIRF dye cyanine7 amine was purchased from Shanghai Seebio Biotech, Inc. Oleic acid (90%), squalane, and 1-octadecene (90%) were purchased from Aldrich. Sodium oleate was obtained from TCI. Iron chloride hexahydrate (FeCl₃•6H₂O), and triethyl phosphate were purchased from Aladdin. Iron (III) chloride (FeCl₃•6H₂O) and trisodium citrate dihydrate (C₈H₅Na₃O₇•2H₂O) were purchased from Shanghai Chemical Reagents Co. Alexa Fluor 594 conjugate was from Molecular Probes (Leiden, The Netherlands). 4′,6-diamidino-2-phenylindole (DAPI), calcein-AM, PI, and Lysotracker Blue were purchased from KeyGen Biotech, Inc. (Nanjing). All chemicals were used as received without further purification. Roswell Park Memorial Institute (RPMI)-1640 and fetal bovine serum were purchased from Gibco (Tulsa, OK).

**Synthesis of OA-Stabilized IONPs**: OA-IONP-5, OA-IONP-11, and OA-IONP-20 were synthesized using a modified literature method.[33]

**Aqueous Phase Transfer of Hydrophobic Magnetic Nanoparticles**: 200 mg of DHCA was dissolved in 24 mL of THF in a three-neck flask (100 mL). The resulting solution was heated to 50 °C under argon flow. Then, 80 mg of hydrophobic magnetic nanoparticles dispersed in 4 mL of THF were added dropwise. After 3 h, the reaction was cooled to room temperature, and 2 mL of 0.5 M NaOH aqueous solution was introduced to precipitate the magnetic nanoparticles. The precipitate was collected by centrifugation (12 000 rpm) and redispersed in 10 mL water for further use.

**Synthesis of IONP-TAT/PEG-Mal**: 20 mg IONP was dissolved in HEPES solution followed by the addition of 25 mg EDC and 25 mg NHS. The mixture was then stirred at room temperature for 24 h to activate the carboxylic group of IONP. Subsequently, 2 mg TAT and 20 µL NH₂-PEG-Mal were added to the above dispersion, and the mixture was stirred for 24 h at room temperature. Excess EDC, NHS, and TAT were removed by repeatedly washing the nanoparticles with distilled water several times. Finally, IONP-TAT/PEG-Mal were dispersed in water and stored at 4 °C.

**Characterization of the Modified Efficiency of Tf**: Tf was modified with sulfydryl groups to obtain Tf-SH. The degree of thiolation of Tf (-SH/Tf) was evaluated using Ellman assay.[66,67] When the reaction ended, IONP-TAT-Tf (IONP-TAT-Tf) was obtained through centrifugation. Based on Ellman assay, the results indicated that 75% of Tf-SH was modified on the surface of IONP-TAT-Tf (Figure S13, Supporting Information).

**Tumor Model**: Male Balb/c nude mice (4 weeks old, ≈20 g body weight) were purchased from Shanghai BK Laboratory Animal Co., Ltd., China. All animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of Fudan University. A549 tumor-bearing mice were established by subcutaneous injection of 2 × 10⁶ cells into the flank region, and in vivo experiment was carried out 2 weeks after the inoculation (when the longest dimension of the tumor reached about 5 mm). The 25 tumor-bearing mice were randomized into five therapy groups (n = 5 per group): PBS, IONP-20, IONP-20-TAT, IONP-20-TAT-Tf, IONP-20-TAT-Mal, and IONP-20-TAT-Mal-Tf. PBS was used as the control group. The average body weight was recorded every 2 d. The tumor growth curve was plotted by the tumor weight of each mouse after being killed (Figure 7).

Figure 7. In vivo photothermal therapy. a) Body weight changes were recorded after therapy every 2 d. b) The tumor growth curves of the mice after treatment. c) Photograph of tumors after excision from different groups under NIR irradiation (808 nm, 3 W cm⁻², 300 s), and tumor weights of each group. d) H&E stained images of major organs of healthy mice and mice 14 d post-IONPs particle injection and photothermal treatment. The scale bar is 200 µm. *p < 0.1, **p < 0.01, ***p < 0.001.
IONP-20-TAT, IONP-20-Tf, and IONP-20-TAT-Tf. Each group of dispersion with a nanoparticle was tail intravenous injected into mice (200 µL, 0.5 mg mL<sup>-1</sup>). The sizes of tumor and mice weight were measured every other day. The tumor volume was calculated by volume = (tumor length)<sup>2</sup> × (tumor length)/2.

In Vivo MRI and NIRF Imaging: Tumor-bearing mice injected with IONP-20-TAT-Tf were used to test the MRI capability of the materials in vivo. Mice were scanned for MR images before or after a certain time of the injection and T<sub>2</sub>-weighted images were obtained by MR scanner (7T, Bruker). The mice were i.v. injected with IONP-20-TAT-Tf at a dose of 5 mg kg<sup>-1</sup>. NIRF imaging was performed using In Vivo Xtreme (Bruker, USA) at different time postinjection. All fluorescent images were acquired with a 10 s exposure time.

Histoogy Analysis: Male Balb/c mice were sacrificed 14 d after i.v. injection and PTT treatment. Tumor and major organs including heart, kidney, liver, lung, and spleen from mice were harvested and fixed in 4% formaldehyde solution. Later, these tumors and organs were processed routinely into paraffin, sectioned at 8 µm thickness, stained with H&E, and examined with a digital microscope.

Statistical Analysis: Data were expressed as mean ± SD, and the statistical significance was determined by using the one-way analysis of variance. Probabilities as p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***). No statistically significant differences were observed.

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

Acknowledgements
This work was financially supported by the State Key Project of Research and Development (Grant No. 2016YFC1100300) and National Natural Science Foundation of China (Grant Nos. 51273047 and 51473037).

Received: November 17, 2016
Revised: December 21, 2016
Published online: January 27, 2017


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