PEG-functionalized iron oxide nanoclusters loaded with chlorin e6 for targeted, NIR light induced, photodynamic therapy

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ABSTRACT

Magnetic targeting that utilizes a magnetic field to specifically deliver theranostic agents to targeted tumor regions can greatly improve the cancer treatment efficiency. Herein, we load chlorin e6 (Ce6), a widely used PS molecule in PDT, on polyethylene glycol (PEG) functionalized iron oxide nanoclusters (IONCs), obtaining IONC–PEG–Ce6 as a theranostic agent for dual-mode imaging guided and magnetic-targeting enhanced in vivo PDT. Interestingly, after being loaded on PEGylated IONCs, the absorbance/excitation peak of Ce6 shows an obvious red-shift from ~650 nm to ~700 nm, which locates in the NIR region with improved tissue penetration. Without noticeable dark toxicity, Ce6 loaded IONC–PEG (IONC–PEG–Ce6) exhibits significantly accelerated cellular uptake compared with free Ce6, and thus offers greatly improved in vitro photodynamic cancer cell killing efficiency under a low-power light exposure. After demonstrating the magnetic field (MF) enhanced PDT using IONC–PEG–Ce6, we then further test this concept in animal experiments. Owing to the strong magnetism of IONCs and the long blood-circulation time offered by the condensed PEG coating, IONC–PEG–Ce6 shows strong MF-induced tumor homing ability, as evidenced by in vivo dual modal optical and magnetic resonance (MR) imaging. In vivo PDT experiment based magnetic tumor targeting using IONC–PEG–Ce6 is finally carried out, achieving high therapeutic efficacy with dramatically delayed tumor growth after just a single injection and the MF-enhanced photodynamic treatment. Considering the biodegradability and non-toxicity of iron oxide, our IONC–PEG–Ce6 presented in this work may be a useful multifunctional agent promising in photodynamic cancer treatment under magnetic targeting.

1. Introduction

Photodynamic therapy (PDT) is a photochemistry-involved treatment process that uses photosensitizers (PS), one kind of light-activatable chemicals, to generate cytotoxic reactive oxygen species (ROS) under light activation, thus causing cell apoptosis and tissue destruction [1]. Different from photothermal therapy (PTT) that employs heat to ‘burn’ cancer [2–6], PDT using much lower optical power densities is noninvasive and can kill cancer cells in a moderate manner [7–10]. On the other hand, compared with chemotherapy and radiotherapy, PDT causes minimal toxicity to normal tissues or organs because the generation of ROS is a light-triggered process and PS agents usually are not toxic in dark [11].

Unfortunately, the poor cancer cell uptake and inefficient tumor delivery of PS agents limit the current applications of PDT in cancer therapy [12]. Moreover, most of currently used PS molecules are excited by visible light with limited tissue penetration. For example, porphyrin-based PS agents widely used in PDT can be excited by red light at 640 nm–660 nm, which could be adsorbed by the blood owing to the existence of hemachrome (also with a porphyrin structure) in red blood cells. Therefore, the development of new PDT agents with enhanced cancer cell uptake, effective tumor homing ability, and are excitable by near-infrared (NIR) light with much better tissue penetration, is still urgently needed.

Delivery of PDT by nanotechnology has received significant attention in recent years. Relying on the “enhanced permeability and retention” (EPR) effect of solid tumors, nanomaterials carrying PS agents are able to passively target tumors to improve the PDT treatment efficacy [13,14]. However, passively delivering the therapeutical agents to solid tumors by EPR effect is not efficient in many
situations because of the pathophysiological heterogeneity of tumors and the huge individual variations in tumor EPR effect [15]. Conjugating of tumor-specific targeting ligands on nano-carriers allows ‘active targeting’ of tumors, and is another extensively explored approach in PDT. Unfortunately, several critical disadvantages, such as the inter-patient variation in receptor expressions, largely limit the widespread clinical applications of molecular tumor targeting [16].

Enhancing tumor accumulation of therapeutic agents by physical forces such as an external magnetic field (MF) has emerged as a new tumor-targeting strategy [17]. During this process, magnetic nanoparticles carrying therapeutics circulating in the bloodstream would be attracted by the MF applied on the tumor, resulting in greatly enhanced enrichment of therapeutic agents in targeted tumor region to improve the cancer treatment efficacy. Compared with passive tumor targeting simply based on the EPR effect, magnetic-targeting guided delivery can further improve the delivery efficiency of therapeutic agents to tumors. Different from active tumor targeting based on the ligand-receptor binding, the performance of magnetic targeting is not constrained by the specific receptor expression, and may be applicable to a wide range of solid tumors regardless of tumor genetic variations. Hitherto, although magnetic tumor targeting for the delivery of chemotherapeutic drugs has been successfully demonstrated [18–25], and several groups have developed magnetic nanoparticles carrying PS molecules for magnetic targeted PDT [26], the application of magnetic targeting for enhance in vivo cancer PDT treatment has been relatively less reported to our best knowledge, except a single earlier report in which the nanomaterials/bioconjugation may not be well optimized [27].

Therefore, in this work, we develop polyethylene glycol (PEG) functionalized iron oxide nanoclusters (IONCs) to load the PS molecule, chlorin e6 (Ce6). The obtained IONC–Ce6 exhibits red-shifted absorption peak, and thus can be excited by the NIR light. Systematic in vitro and in vivo experiments are designed to carefully evaluate the cellular uptake, light-induced cell killing, blood circulation, biodistribution, as well as magnetic tumor targeting and MF-enhanced PDT of those IONC–Ce6 nanoparticles at both cellular and animal levels. Different from the previously reported magnetic nanoparticle-based Ce6 delivery system [27], our IONC–Ce6 with well engineered surface chemistry enables NIR-triggered PDT, and demonstrates excellent PDT efficacy in vitro and in vivo under magnetic targeting. Our work presents a promising multifunctional theranostic nano-agent based on iron oxide, which has been demonstrated to be biodegradable and non-toxic, for MF-enhanced photodynamic cancer treatment.

2. Experimental section

2.1. Chemicals

Ferric chloride hexahydrate (FeCl3·6H2O), sodium acetate (CH3COONa, NaOAc), ethanol, ethylene glycol (EG), diethylene glycol (DEG) and polyvinylpyrrolidone (PVP, K-30) were purchased from Sinopharm Chemical Reagent Co., Ltd. Dopamine (DA) and polyacrylic acid (PAA) were obtained from Sigma–Aldrich. Amino group terminated polyethylene glycol (PEG-NH2, 5K) was purchased from Aladdin. Chlorin e6 (Ce6) was the product of J&K Chemical Co.

2.2. Synthesis of IONCs and surface modification

IONCs were synthesized using a solvent thermal method. FeCl3·6H2O (2 mmol) was dissolved in the mixture of 6 mL ED and 14 mL DEG. After magnetic stirring for 30 min, 2 g PVP was added into the solution and heated at 125 °C for 1 h and then 1.5 g NaOAc was added. After stirring for another 0.5 h, the final suspension was transferred to a Teflon-lined stainless-steel autoclave (20 mL), which was heated at 200 °C for 12 h. The obtained IONCs were washed with ethanol and water three times. The final product was dispersed in 15 mL tetrahydrofuran to prepare a stock solution with the concentration of 8 mg/mL.

18 mg PAA (0.01 mmol) and 625 mg PEG-NH2 (0.125 mmol) were dissolved in 2 mL dimethylformamide (DMF), into which 95.85 mg 1-ethyl-3-(3-dimethylamino)propyl) carbodiimide hydrochloride (EDC, 0.5 mmol) and 104.5 mg triethylamine (TEA) were added to the solution. The mixture was stirred for 24 h at room temperature under the protection of nitrogen. After adding 76.59 mg DA (0.5 mmol), 95.85 mg EDC (0.5 mmol) and 139 mL TEA, the final mixture was stirred for another 24 h under the same condition. The obtained suspension was dialyzed against deionized water using dialysis membrane (MWCO 10,000–14,000) for 24 h. The solution was frozen dried, yielding the final product DA–PAA–PEG copolymer in a white solid.

To functionalize IONCs, 50 mg of DA–PAA–PEG was dissolved in 5 mL deionized water, into which 2.5 mL stock solution of IONCs was added under sonication. The sonication was continued for 50 min with temperature kept below 25 °C. The mixture was stirred overnight. Magnetic separation was carried out to remove excess DA–PAA–PEG. After being washed with water for three times, IONC–PAA–PEG was dispersed in 10 mL deionized water for future use.

2.3. Ce6 loading on IONC–PAA–PEG

Generally, Ce6 used during this experiment was pre-dissolved in dimethyl sulfoxide (DMSO). 0.2 mg of PEGylated IONCs and 0.05 mL Ce6 (20 mg/mL) were mixed in 1 mL phosphate buffered saline (PBS) with different pH value (6.6, 7.4 and 8.0). The mixture was stirred overnight under room temperature. Free Ce6 was removed by magnetic separation, which was carried out several times until the supernatant after separation became colorless. To measure the loading capacity, 0.1 mL Ce6 solution with different concentrations (1, 5, 10, 15 and 20 mg/mL) was added into 0.9 mL PBS (pH = 8.0) containing 0.2 mg IONC–PAA–PEG. After shaking overnight at room temperature, excess Ce6 was removed by the same method as described above. To determine the Ce6 loading on IONC–PAA–PEG, 0.5 mL HCl (5 μL) was added into the obtained IONC–PAA–PEG solution to decompose Fe3O4 and release Ce6. UV/Vis/NIR absorbance spectrum was used to determine the concentration of Ce6 based on the molar extinction coefficient of Ce6 in HCl at 645 nm (2.33 × 104 M−1 cm−1). To block the carbonyl groups of Ce6, 88.5 mg propylamine (1.5 mmol), 60 mg Ce6 (0.1 mmol) and 278 mg EDC (1.5 mmol) were dissolved in 0.5 mL DMSO. After reacting for 24 h, adjust the pH to 5.0 to precipitate blocked Ce6. Then, the final product was washed with PBS (pH = 5.0) three times. The loading experiment was conducted as described previously.

2.4. Characterization of the prepared nanomaterials

The morphology and structure of IONCs were characterized by scanning electron microscopy (SEM) images using a FEI Quanta 200F scanning electron microscope and Transmission electron microscopy (TEM) images using a Philips CM300 transmission electron microscope. UV/Vis/NIR spectra were recorded on PerkinElmer Lambda 750 UV/Vis/NIR spectrophotometer. Fluorescent spectra of free Ce6 and IONC–PAA–PEG were measured by FluoroMax 4 luminescence spectrometer (HORIBA Jobin Yvon).

2.5. Detection of singlet oxygen

In our experiments, we used the light source in the Maestro in vivo animal imaging system to trigger PDT. Different samples were exposed to the light with central wavelengths at 660 nm and 704 nm passing the respective band-pass filters. The power density was measured by an optical power meter (LPE-1C, PhysioScience Opto-Electronics, Beijing) to be 5 mW/cm² for light at both wavelengths. Singlet oxygen sensor green (SOSG), which was highly sensitive for singlet oxygen, was employed here during detection process. Typically, SOSG under the concentration of 2.5 μM was introduced to measure the SO generation of Ce6 (0.25 μM) loaded IONC–PAA–PEG under light irradiation at 660-nm and 704-nm wavelengths. Control groups include SOSG alone, free Ce6, and bare IONC–Ce6 without Ce6 loading. The generated SO was determined by measuring recovered SOSG fluorescence of SOSG (excitation = 494 nm).

2.6. Cellular uptake assay

Murine breast 4T1 cancer cells were cultured in RPMI-1640 medium containing 10% FBS and 1% penicillin/streptomycin at 37 °C under 5% CO2. 4T1 cells (1 × 105 cells) were cultured in 35 mm culture dishes containing 40 μg/mL IONC–PAA–PEG or 2.6 μg/mL free Ce6 in the dark for different periods of time (0.5, 2, 6, 12 and 24 h). After washing with PBS (pH = 7.4) for three times, cells were labeled with 4,6-diamidino-2-phenylindole (DAPI) and then imaged by a laser scanning confocal fluorescence microscope (Leica SP5). To quantitatively determine cellular uptake of Ce6, cells after incubation and washing were lysed with 0.5 mL of 2% sodium dodecyl sulfate (SDS) for 2 h and then treated with 0.5 mL HCl (5 μL) for 12 h. Fluorescence spectra of the cell lysate solutions were measured under 404 nm excitation to determine Ce6 concentrations.
2.7. In vitro PDT and magnetic targeting

For in vitro PDT experiments, 4T1 cells (1 x 10^5) seeded in 96-well plates were added with IONC–PEG–Ce6, free Ce6 and IONC–PEG at various concentrations. After incubation for 12 h, the experimental groups were exposed to 661-nm (for Ce6) or 704-nm (for IONC–PEG–Ce6 and IONC–PEG) light irradiation under a power density of 5 mW/cm² for 30 min, while the control groups were still cultured in dark. Afterwards, all samples were incubated in dark for another 12 h. In order to determine relative cell viabilities after various treatments, the MTT (Sigma Inc.) assay was conducted followed the standard protocol. 

2.8. Tumor model

Balb/c mice weighing 18-20 g were purchased from Suzhou Belda Bio-Pharmaceutical Co., Ltd. and used in accordance with regulations provided by Soochow University Laboratory Animal Center. 4T1 tumors were inoculated by subcutaneous injection of 5 x 10^5 cells in ~100 μL of serum-free RPMI-1640 medium onto the back of female Balb/c mice.

2.9. In vivo behavior

Five mice were i.v. injected with IONC–PEG–Ce6 (5 mg/mL, 200 μL for each mouse). About 10 μL blood was extracted from the tail at different time points and then dissolved in 0.5 mL lysis buffer (15 SDS, 1 Triton X-100, 40 ms Tris acetate). 0.5 mL HCl was added to decompose IONCs and release Ce6. After centrifugation at 1000 rpm for 5 min, Ce6 fluorescence in the blood samples was measured. After subtracting auto-fluorescence determined by measuring the blood sample collected from an untreated mouse, the blood concentrations of Ce6 were then calculated and presented by unit of the percentage of injected dose per gram tissue (%ID/g).

2.10. In vivo magnetic targeting and PDT

For in vivo PDT, tumors from IONC–PEG–Ce6 were injected mice with or without magnetic tumor targeting were exposed to the 704-nm light at 5 mW/cm² for 1.5 h. As the control, tumors on Ce6 injected mice were irradiated by the 661-nm light also at 5 mW/cm² for 1.5 h. Six mice were used in each group. The lengths and widths of tumors in different groups were measured every two days after treatment. The tumor volume was calculated by (length of tumor) x (width of tumor)^2/2.

3. Results and discussion

3.1. Preparation and characterization of IONC–PEG–Ce6

The schematic illustration to show the preparation of Ce6 loaded IONC–PEG is shown in Fig. 1a. IONCs were synthesized using the binary solvothermal method following a literature protocol [28]. As revealed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Fig. 1b), the as-made IONCs showed rather uniform morphology and sizes with an average diameter of ~100 nm. Being clusters of ultra-small iron oxide nanocrystals, IONCs with rough surface exhibited a large specific surface area, potentially allowing efficient loading of therapeutic molecules. In order to understand the crystalline nature of the nanocrystals, the powder X-ray diffraction (XRD) was carried out, suggesting the cubic crystalline structure of IONCs (Supporting Fig. S1).

The magnetic property of PEGylated IONCs was then studied. As-prepared IONCs exhibited superparamagnetic property owing to the absence of a hysteresis loop in the field-dependent magnetization measurement (Fig. 1c). After being exposed to an external magnetic field, IONC–PEG was rapidly attracted to one side nearby the magnet (Fig. 1c, inset). T2-weighted MR imaging revealed a concentration-dependent darkening effect with the transverse relaxivity coefficient (r₂) measured to be 85.48 mM⁻¹ s⁻¹ (Fig. 1d).

Dopamine (DA) with two phenol groups is a commonly used molecule to modify the surface of iron oxide [29-31]. To functionalize IONCs, we designed and synthesized a polymer by co-grafting polyacrylic acid (PAA) with DA and PEG-NH₂ (5 kDa). The successful synthesis of DA–PAA–PEG was proved by the 1H NMR spectrum of the final product (Fig. S2). Showing 42% and 23% of carboxyl groups on the backbone of PAA conjugated to PEG-NH₂ and DA via amide bonds, respectively. Due to the improved orbital overlap of the five-member ring, DA could strongly coordinate to the Fe atom on surface of IONCs by Fe–O bonds [32]. Notably in our system, DA–PAA–PEG contains multiple DA molecules, which could bind to the IONCs surface super strongly as the result of the poly-valance effect, offering significantly enhanced functionalization stability compared to IONCs functionalized by single-chain DA–PEG (Fig. 2a) [33]. As expected, our PEGylated IONCs possess brilliant solubility and stability in various physiologic solutions (Fig. 2b). To the contrary, IONCs without surface modification precipitated quickly in aqueous solutions, resulting in the larger diameter and broad size distribution as confirmed in Fig. 2c. Both Fourier transform infrared (FT-IR) spectra (Supporting Fig. S3) and size distribution of IONCs before and after PEGylation directly evidenced the successful PEG functionalization in IONC–PEG.

To test Ce6 loading on PEGylated IONCs, we mixed IONC–PEG with Ce6 under constant shaking overnight at pH = 8.0. After removal of excess Ce6 by magnetic separation, UV/Vis/NIR spectra were recorded. Notably, the Ce6 absorbance peak at ~650 nm shifted to ~700 nm, and its absorbance at 404 nm largely diminished after being loaded onto the surface of IONC–PEG (Fig. 3a), implying a chemical bonding instead of simple physical adsorption might have happened between Ce6 and IONC. Similar to the binding of DA on the iron oxide surface, we speculated that the three carboxyl groups in a Ce6 molecule might form coordinate bonds with the Fe atom on the IONC surface (Fig. 1a). To test this hypothesis, the carboxyl groups of Ce6 were blocked by propylamine via amide bonds, obtaining carboxyl-blocked Ce6 which was introduced for loading experiment (Supporting Fig. S4a) conducted under the same experimental condition. Once carboxyl groups of Ce6 were blocked, no appreciable Ce6 loading on IONC–PEG was observed, suggesting that the carboxyl groups of Ce6 should play an important role in terms of Ce6 loading on IONCs. We also noticed that the loading of Ce6 on IONC–PEG was pH-dependent and showed increased loading capacity under basic pH (Supporting Fig. 54b), indicating that the de-protonation of carboxyl groups was helpful for Ce6 loading. Considering the fact that the hydrophobicity of Ce6 would decrease as the rise of pH, it is reasonably to believe that it should indeed be the interaction between Ce6 carboxyl groups and iron oxide surface, instead of the simple hydrophobic adsorption, that attributes to the effectively Ce6 loading on PEGylated IONCs. Last but not least, the loading of Ce6 didn’t affect the solubility and stability of IONC–PEG because no obvious precipitation was observed in Fig. 2b and size distribution before and after Ce6 loading didn’t change too much (Fig. 2c).
We then evaluated the Ce6 loading capacities on IONC–PEG. To get the loading curve, Ce6 was loaded onto IONC–PEG at different feeding concentrations in phosphate buffer (pH 8.0), with excess free Ce6 removed after loading. Because of the significant change in optical absorbance of Ce6 after being adsorbed on IONCs, it was not possible to determine the precise Ce6 loading directly based on the absorbance spectra of IONC–PEG–Ce6 shown in Fig. 3a. To quantitatively determine the amount of Ce6 loaded in IONC–PEG–Ce6, a hydrochloride (HCl) solution (5 M) was added to dissolve iron oxide cores and release Ce6, whose concentration was then determined by its absorbance at 645 nm based on a standard calibration curve (diluting Ce6 with known concentrations in HCl, Supporting Fig. S5). Finally as shown in Fig. 3b, as the rise of added Ce6 concentrations, the amounts of Ce6 adsorbed on IONC–PEG–Ce6 increased. The Ce6 loading approached to saturation with the loading ratio of 6.5% (w/w) at the feeding Ce6 concentration of 1 mg/mL. IONC–PEG–Ce6 prepared under this condition was used for our further experiments.

The stability of IONC–PEG–Ce6 was tested by incubating IONC–PEG–Ce6 in phosphate buffers (pH = 5.0, 7.4 and 9.0) and FBS for two days, and then measuring the amounts of released Ce6 (Supporting Fig. S6). It was found that the Ce6 loading on PEGylated IONCs was rather stable in serum and buffers with neutral or alkaline pH. Slightly increased Ce6 release was noted for IONC–PEG–Ce6 incubated at pH 5.0, likely owing to the protonation of carboxyl groups in Ce6 that weakened its binding with the iron oxide surface.

Using IONC–PEG–Ce6 prepared under the optimized condition, we studied the excitation behaviors of IONC–PEG–Ce6 in comparison to free Ce6 using an in vivo fluorescence imaging system (Maestro EX). Consistent to the red-shift of the absorbance peak, we found that the fluorescence excitation of IONC–PEG–Ce6 also greatly shifted to the longer wavelength (Fig. 3c). Such a red-shift of excitation wavelength from 660 nm to 704 nm is beneficial for in vivo fluorescence imaging because of the low tissue absorbance and scattering in the NIR window from 700 nm to 900 nm. We then wondered whether the PDT excitation wavelength, under which PS molecules produce ROS such as singlet oxygen (SO), would also show a red-shift. The generation of ROS was detected using the singlet oxygen sensor green (SOSG), whose quenched fluorescence in the aqueous solution could be recovered in the presence of SO. The light passing through a specific band-pass filter from the in vivo fluorescence imaging system was utilized as the excitation light source with centered wavelength at 661 nm or 704 nm. Indeed, while the SO generation for free Ce6 was effective under 661-nm excitation, it became barely detectable if the wavelength was switched to 704 nm (Fig. 3d). In marked contrast, IONC–PEG–Ce6 offered obviously improved SO generation under the longer wavelength excitation at 704 nm.

3.2. In vitro experiments

Although several formulations of functionalized iron oxide nanoparticles have already been used for clinical MR diagnosis for many years, we still need to test the potential toxicity of our PEGylated IONCs to be cautious. Cell viability test based on the standard thiazolyl blue tetrazolium bromide (MTT) assay (Fig. 4) revealed that our IONC–PEG exhibited no obvious cytotoxicity to several different types of cells, including 4T1 murine breast cancer cells, human embryonic kidney 293 cells (293T) and mouse embryonic fibroblast cells (NIH 3T3), even under high concentrations up to 200 μg/mL.
We next studied the cell entry of IONC-PEG-Ce6 in comparison to free Ce6 by confocal fluorescence microscopy. As shown in Fig. 5a, strong Ce6 red fluorescence signals emerged in cytoplasm of 4T1 cells incubated with IONC-PEG-Ce6 by a time-dependent manner, indicating the efficient cellular uptake of Ce6 loaded nanoparticles by endocytosis. In contrast, the Ce6 fluorescence signals in cells incubated with free Ce6 at the same concentration were much weaker. Considering the significant Ce6 fluorescence quenching after loading on PEGylated IONCs, the actual amount of Ce6 being shuttled into cells using IONC-PEG-Ce6 should be much

![Image]

**Fig. 2.** PEG functionalization of IONCs. (a) Photos of aqueous solutions of IONCs modified by DA-PAA-PEG or DA-PEG taken at different days. DA-PAA-PEG modified IONCs exhibited remarkably improved stability in comparison to IONCs coated by DA-PEG due to the multi-valence effect. (b) Photos of as-made IONCs, IONC-PEG and IONC-PEG-Ce6 culturing in water, PBS (pH = 7.4), cell medium and FBS for 1 day. (c) Dynamic light scattering of synthesized nanomaterials. PEGylation could reduce aggregation of as-made IONCs in the aqueous solution. The loading of Ce6 would not affect the dispersity of IONC-PEG.

![Image]

**Fig. 3.** Ce6 loading on IONC-PEG. (a) UV/Vis/NIR spectra of IONC-PEG, free Ce6 and IONC-PEG-Ce6 at different feeding Ce6 concentrations. (b) Ce6 loading weight ratios obtained at different feeding concentrations (pH = 8.0). The concentration of IONC-PEG was fixed at 0.2 mg/mL in this experiment. Error bars were based on standard deviations (SD) of triplicated samples. (c) Fluorescence images of IONC-PEG, IONC-PEG-Ce6 and Ce6 aqueous solutions at different excitation wavelengths (661 nm and 704 nm). The exposure time was 100 ms. (d) The fluorescence intensity changes of SOSG as the functional of light exposure time (5 mW/cm²). The increase of SOSG fluorescence was a result of SO generation. SOSG dissolved in water under the concentration of 2.5 μM was introduced to measure the SO generation of Ce6 loaded IONC-PEG under light irradiation at 660-nm and 704-nm wavelengths.
higher than that of free Ce6 itself. In order to quantitatively determine the cellular uptake of Ce6, cells incubated with IONC–PEG–Ce6 or Ce6 were incubated by a lysis buffer and then treated with an HCl solution to allow complete decomposition of IONCs and the release of loaded Ce6. Fluorescence intensities of cell lysates for samples collected at varied time points further confirmed that the cellular uptake of IONC–PEG–Ce6 was much more efficient than that of free Ce6, showing an enhancement factor as high as 17.5-fold at 24 h post-incubation (Fig. 5b). This kind of significantly enhanced Ce6 delivery may likely be owing to the effective endocytosis of PEGylated IONCs by cells compared with the poor cross-membrane diffusion ability of free Ce6 molecules [40].

The phototoxicity of IONC–PEG, free Ce6 and IONC–PEG–Ce6 to 4T1 cells was then determined using the MTT assay. Without light exposure, Ce6 and IONC–PEG–Ce6 exhibited negligible dark toxicity to 4T1 cells as evidenced in Fig. 5c. Under 704-nm light exposure, IONC–PEG–Ce6 showed remarkable and concentration-dependent phototoxicity (Fig. 5d), which was much stronger than that of free Ce6 under 661-nm light irradiation at the same optical dose (5 mW/cm² for 30 min, or 9 J/cm²). Since the phototoxicity of bare IONP–PEG was negligible, the improved PDT potency of IONC–PEG–Ce6 over free Ce6 could mainly be attributed to the dramatically enhanced cellular Ce6 uptake for cells incubated with IONC–PEG–Ce6.

Before studying in vivo magnetic tumor targeting using IONC–PEG–Ce6, we first tested its blood circulation behavior after intravenous (i.v.) injection into mice. Blood was extracted from Balb/c mice received i.v. injected IONC–PEG–Ce6 (dose: 50 mg/kg) at various time points post-injection (p.i.). After adding lysis buffer to homogenize blood samples and HCl to decompose IONCs, fluorescence spectra were recorded to determine the concentrations of IONP–PEG–Ce6 in the blood based on Ce6 fluorescence. As shown in Fig. 7b, the blood level of IONC–PEG–Ce6 decreased gradually over time in accordance with a two-compartment model. The first ($t_{1/2}$) and second ($t'_{1/2}$) phase of circulation half-lives were calculated to be 0.30 ± 0.04 h and 3.78 ± 0.29 h respectively by secondary exponential fitting. The fairly long circulation time of IONC–PEG–Ce6 in the blood would allow for effective magnetic tumor targeting, in which a magnetic field is applied to attract magnetic nanoparticles circulating in the blood to the tumor region, where the blood vasculature is disordered and thus tend to trap nano-sized particles.
In vivo fluorescence/MR dual-modal imaging was then conducted to track IONC–PEG–Ce6 nanoparticles after being injected into tumor-bearing mice which were placed under a magnetic field.

In our experiments, Balb/c mice each bearing two 4T1 murine breast cancer tumors on both sides were i.v. injected with IONC–PEG–Ce6 (200 μL, 5 mg/mL), with their tumors on the right side attached to a magnet (3000 G) (Fig. 7a). In vivo fluorescence image revealed that the tumor on the right side with a magnet attached
showed rather strong Ce6 fluorescence, while that on the left side tumor in the absence of the magnetic field was much weaker (Fig. 7c), indicating the highly effective magnetic field induced tumor homing of IONC–PEG–Ce6 nanoparticles. T2-weighted MR imaging further confirmed the prominent accumulation of IONC–PEG–Ce6 in the tumor exposed to the magnetic field (Fig. 7d). Quantitative analysis of fluorescence imaging data revealed that the magnetic targeting could enhance the Ce6 fluorescence in the tumor region by ~8.9 fold (Fig. 7e). As for the MR images, the averaged T2 signal intensity decreased by 11.0% or 94.4% in tumors without or with magnetic targeting, respectively, post-injection of IONC–PEG–Ce6 (Fig. 7f).

Those mice were then sacrificed at 24 h p.i. for biodistribution study. After dissolving all collected organs and tissues by aqua regia, the inductively coupled plasma atomic emission spectroscopy (ICP-AES) measurement was conducted to determine Fe$^{3+}$. 

Fig. 7. In vivo magnetic tumor targeting. (a) A schematic drawing to illustrate in vivo magnetic tumor targeting. (b) Blood circulation curve of IONC–PEG–Ce6 in mice by measuring the concentration of IONC–PEG–Ce6 in blood at different time points post-injection. Error bars were based on triplicated samples. (c) In vivo fluorescence image of a 4T1 tumor bearing mouse. (d) In vivo T2-weighted MR images of a mouse taken before injection (upper) and 24 h post injection (bottom). White and red arrows point to tumors without and with a magnet attached, respectively. (e) Ce6 fluorescence signal intensities in magnetic field (MF) targeted and non-targeted tumor regions. (f) T2-weighted MR signals of untreated, MF targeted and non-targeted tumors. (g) Biodistribution of IONC–PEG–Ce6 in mice under magnetic targeting for 24 h after i.v. injection. The concentration of Fe$^{3+}$ was measured by ICP-AES. Error bars were based on SD of three mice. (h) Prussian blue stained images of major organs. Blue spots indicated the presence of IONCs. The number of blue spots in the MF targeted tumor was much more than that in the non-targeted tumor, demonstrating the enhanced tumor enrichment of IONC–PEG–Ce6 under magnetic targeting. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
levels in those samples (Fig. 7g). Organs samples collected from an untreated mouse were also measured to determine the background Fe$^{3+}$ levels in different organs. Similar to the biodistribution profiles of the majority of nanomaterials [41–43], significant accumulation of IONC–PEG–Ce6 was observed in reticuloendothelial systems including liver and spleen, reaching ~25%ID/g and ~10% ID/g, respectively. Importantly, the enrichment of IONC–PEG–Ce6 in tumors exposed to the external magnetic field reached to ~13% ID/g, which was ~3.5-fold higher compared to the passive accumulation of nanoparticles in the non-targeted tumors. The Prussian blue stained images also confirmed the enhanced enrichment of IONC–PEG–Ce6 in magnetic-field targeted regions compared with non-targeted tumor (Fig. 7h). Although the exact enhancement factor showed certain variation due to difference in measurement approaches, our data clearly demonstrated the highly effective magnetic tumor targeting ability of IONC–PEG–Ce6.

3.4. In vivo magnetically targeted PDT

Being aware of the high NIR-induced SO generation efficiency of IONC–PEG–Ce6, the efficient cancer cell uptake of those nanoparticles, and the excellent in vivo magnetic tumor targeting effect, we finally carried out magnetically targeted PDT treatment study using the 4T1 tumor modal on Balb/c mice. Six mice each bearing two tumors were intravenously injected with 200 μL IONC–PEG–Ce6 (5 mg/mL of IONC, 0.325 mg/mL of Ce6). The tumor on the right side of each mouse was attached to a magnet for 24 h and while the other tumor was not. Both tumors on each mouse were then exposed to 704-nm light irradiation (in the Maestro in vivo imaging system) for 1.5 h at a power density of 5 mW/cm$^2$ (27 J/cm$^2$). Another six mice also injected with IONC–PEG–Ce6 at the same dose but without light exposure were used as a control. For comparison purpose, six tumor-bearing mice injected with free Ce6 (200 μL, 0.325 mg/mL) were irradiated by 661-nm light (5 mW/cm$^2$, 1.5 h, 27 J/cm$^2$). The tumor sizes were measured every two days after treatment. As shown in Fig. 8a and b, PDT treatment using free Ce6 at the current injection dose and optical dose showed marginal effect in delaying the tumor growth. The tumors on IONC–PEG–Ce6 injected mice in the absence of the magnetic field showed partially reduced growth only at the early time points, and grew rapidly after ~1 week post-light irradiation. Excitingly, the growth of tumors on the same group mice but exposed to the magnetic field were greatly inhibited after light irradiation, showing little tumor volume increase over a course of 16 days post-PDT treatment. As expected, tumors of mice receiving IONC–PEG–Ce6 injection without light exposure showed the same growth trend as the untreated groups, indicating that IONC–PEG–Ce6 itself in dark would not affect the tumor development. These results demonstrated that magnetic targeting could greatly enhance the PDT efficacy by increasing the enrichment of PS molecules (Ce6) shuttled by the nanocarrier (IONC–PEG–Ce6) into the targeted tumor region under an external magnetic field.

Several formulations of iron oxide nanoparticles have been approved by FDA as MR contrast agents for clinical use [44]. In our...
experiments, neither body weight loss nor death was observed for IONC–PEG–Ce6 injected mice (Supporting Fig. S7). All mice behaved normally after treatment. Hematoxylin and eosin (H&E) stained images of main organs from IONC–PEG–Ce6 injected healthy mice collected 20 days after injection also suggest that our Ce6 carrying PEGylated IONCs did not induce appreciable toxic side effects to treated animals (Fig. 8c).

4. Conclusion

In summary, a new type of magnetic nanocarrier based on PEGylated IONCs is successfully fabricated to specifically deliver photodynamic therapeutic reagents to targeted tumor regions in response to an external magnetic field. In our system, the graft copolymer, DA–PAA–PEG, can strongly bind to the surface of IONCs by the poly-valence effect, allowing condensed PEG coating that greatly improves the physiological stability of our nanoparticles. The large specific surface area of IONCs offers plenty space for Ce6 loading. The red-shift of Ce6 absorbance/excitation peak from red to optical imaging and phototherapy. Because of the greatly accelerated cellular uptake, IONC–PEG–Ce6 exhibits much stronger PDT cancer cell killing potency in comparison to free Ce6. Remarkable in vivo magnetic tumor targeting of IONC–PEG–Ce6 is then uncovered by in vivo dual modal fluorescence/MR imaging, which reveals the highly effective tumor homing ability of our magnetic PDT nano-agent guided by the external magnetic field. Most importantly, outstanding in vivo cancer therapeutic efficacy is achieved using IONC–PEG–Ce6 as the photodynamic agent under magnetic control after light irradiation at a low optical dose. Considering that the PEGylated IONCs are non-toxic and biodegradable, this new magnetic photodynamic nano-agent serving as a magnetic responsive theranostic agent may indeed have great potential in clinical PDT cancer treatment.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.08.041.

References


