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

Zhiwei Li [a], Chao Wang [a], Liang Cheng [a], Hua Gong [a], Shengnan Yin [b], Qiufang Gong [a], Yonggang Li [b], Zhuang Liu [a,*]

[a] Jiangsu Key Laboratory for Carbon-Based Functional Materials & Devices, Institute of Functional Nano & Soft Materials Laboratory (FUNSOM), Soochow University, Suzhou, Jiangsu 215123, China
[b] Department of Radiology, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215006, China

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ABSTRACT

Magnetic targeting that utilizes a magnetic field to specifically delivery 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 therapeutic agents to solid tumors by EPR effect is not efficient in many

* Corresponding author.
E-mail addresses: zliu@suda.edu.cn, zliu828@gmail.com (Z. Liu).

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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–PEG–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–PEG–Ce6 nanoparticles at both cellular and animal levels. Different from the previously reported magnetic nanoparticle-based Ce6 delivery system [27], our IONC–PEG–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 ($FeCl_3 \cdot 6H_2O$), sodium acetate (CH_3COONa, 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 poly(ethylene glycol) (PEG-NH_2, 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. $FeCl_3 \cdot 6H_2O$ (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-NH_2 (0.125 mmol) were dissolved in 2 mL dimethylformamide (DMF), into which 95.85 mg 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 0.5 mmol) and 104.5 μL 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 μL 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–PEG was dispersed in 10 mL deionized water for future use.

2.3. Ce6 loading on IONC–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 shaked 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, 2, 5, 10, 15 and 20 mg/mL) was added into 0.9 mL PBS (pH = 8.0) containing 0.2 mg IONC–PEG. After shaking overnight at room temperature, excess Ce6 was removed by the same method as described before. To determine the Ce6 loading on IONC–PEG, 0.5 mL HCl (5 M) was added into the obtained IONC–PEG–Ce6 solution to decompose Fe_3O_4 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 (2.33×10^4 M^{-1} cm^{-1}). To block the carboxyl 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 carried out using PerkinElmer Lambda 750 UV/Vis/NIR spectrophotometer. Fluorescent spectra of free Ce6 and IONC–PEG–Ce6 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, Physcience Opto-Electronics, Beijing) to be 5 mW/cm^2 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–PEG under light irradiation at 660-nm and 704-nm wavelengths. Control groups include SOSG alone, free Ce6, and bare IONC–PEG 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% CO_2. 4T1 cells (1×10^5 cells) were cultured in 35 mm culture dishes containing 40 μg/mL IONC–PEG–Ce6 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 lyzed with 0.5 mL of 2% sodium dodecyl sulfate (SDS) for 2 h and then treated with 0.5 mL HCl (5 M) for 12 h. Fluorescence spectra of the cell lysate solutions were measured under 404 nm excitation to determine Ce6 concentrations.

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