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Protein modified upconversion nanoparticles for imaging-guided combined photothermal and photodynamic therapy

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ABSTRACT

In this work, we develop a multifunctional nano-platform by coating upconversion nanoparticles (UCNPs) with bovine serum albumin (BSA), obtaining UCNP@BSA nanoparticles with great solubility and stability in physiological environments. Two types of dye molecules, including a photosensitizer, Rose Bengal (RB), and an NIR-absorbing dye, IR825, can be simultaneously loaded into the BSA layer of the UCNP@BSA nanoparticles. In this carefully designed UCNP@BSA-RB&; IR825 system, RB absorbs green light emitted from UCNPs under 980-nm excitation to induce photodynamic cancer cell killing, while IR825 whose absorbance shows no overlap with upconversion excitation and emission wavelengths, offers nanoparticles a strong photothermal perform under 808-nm laser irradiation. Without showing noticeable dark toxicity, the obtained dual-dye loaded nanoparticles are able to kill cancer via combined photothermal and photodynamic therapies, both of which are induced by NIR light with high tissue penetration, by a synergetic manner both *in vitro* and *in vivo*. In addition, the intrinsic paramagnetic and optical properties of Gd^{3+}-doped UCNPs can further be utilized for *in vivo* dual modal imaging. Our study suggests that UCNPs with well-designed surface engineering could serve as a multifunctional nano-platform promising in cancer theranostics.

1. Introduction

Conventional cancer therapies including chemotherapy and radiotherapy have many limitations such as toxic side effects and drug resistance, and often fail to completely eradicate the tumor. Phototherapy is a class of non-invasive therapeutic techniques with many advantages such as remote controllability, improved selectivity, and low systemic toxicity [1]. Photothermal therapy (PTT) and photodynamic therapy (PDT) are two different types of phototherapy methods. PTT involves optical absorbing agents, such as gold nanostructures (nanoshells, nanorods, nanostars and nanocages) [2–5], carbon nanomaterials (carbon nanotube and graphene) [6,7], and various other inorganic [8] and organic nanoparticles [9,10] with strong near infrared (NIR) absorbance, to effectively convert the photo energy into heat to kill cancer cells under light irradiation [10–12]. On the other hand, PDT includes three key components: light, photosensitizer molecules and oxygen [13,14]. Under the appropriate light irradiation, the photosensitizers will transfer the absorbed optical energy to surrounding oxygen molecules, generating cytotoxic singlet oxygen ($^1O_2$) or reactive oxygen species (ROS) to kill cancer cells. Ideal phototherapy agents should exhibit minimal dark toxicity and are effective in cancer destruction under light exposure, via either photothermal or photodynamic mechanisms.

Light penetration is one of major challenges in phototherapy. The NIR window in the range of 700–1000 nm, in which biological tissues have the minimal light absorption, is ideal for optical imaging [15,16] and phototherapy. Although NIR light is commonly used in PTT, unfortunately, most photosensitizers in current PDT are excited by UV or visible light, which has limited tissue penetration depth, limiting the therapeutic efficacy of PDT for large or deep tumors [17–20]. Upconversion nanoparticles (UCNPs) usually containing rare-earth elements under the NIR excitation can emit UV-visible light [21], which can in turn active photosensitizers absorbed on their surface via resonance energy transfer, generating

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reactive $^1O_2$ or ROS to kill cancer cells [22–28]. Hitherto, we and others have used UCNPs coupled with photosensitizers for photodynamic therapy, such as using Chlorin e6 (Ce6) adsorbed/conjugated UCNPs to realize highly effective NIR-induced *in vitro* and *in vivo* PDT therapy [29–32]. Moreover, by coating UCNPs with gold or silver shells or graphene oxide, the obtained nanocomposites could also serve as photothermal agents for cancer cell ablation *in vitro* and *in vivo* [33,34]. However, the combination of NIR-induced photodynamic and photothermal therapy based on UCNPs to realize synergistic *in vivo* anti-tumor effect has not yet been reported to our best knowledge.

In this work, we use a protein, bovine serum albumin (BSA), to modify $NaGdY_4$-based UCNPs. The obtained UCNP@BSA nanoparticles show excellent solubility in water as well as physiological solutions. Utilizing the hydrophobic domains in the BSA protein, two different dye molecules, including a photosensitizer, Rose Bengal (RB) [27], and an NIR-absorbing dye, IR825, can be effectively loaded onto UCNP@BSA nanoparticles. The obtained dual-dye loaded nanoparticles on one hand could serve as a dual modal magnetic resonance (MR) and upconversion optical imaging probe, on the other hand are effective in both photodynamic and photothermal therapy, which if combined together could result in excellent synergetic cancer killing effects both *in vitro* and *in vivo*. This is the first demonstration of using UCNPs for *in vivo* imaging-guided combined photodynamic and photothermal therapies, both of which are induced by NIR light with high tissue penetration.

2. Materials and methods

2.1. Materials

All chemicals were analytical grade and used without further purification. Gd_2O_3, Yb_2O_3, Er_2O_3 and trifluoroacetic acid (CF_3COOH) were purchased from shanghai chemical industrial Co. All the trifluoroacetates were prepared by dissolving the respective rare-earth oxides in trifluoroacetic acid (CF_3COOH). Sodium trifluoroacetate, oleic acid (OA, 90%), 1-octadecene (ODE >90%), poly(acrylic acid) (PAA MW = 1800) and Rose Bengal were purchased from Sigma–Aldrich. Bovine serum albumin (BSA) was purchased from J&C chemical CO. Triethylamine (TEA), diethylene glycol (DEG) were purchased from Sinopharm Chemical Reagent Co. IR825 dye was synthesized following our previously reported protocol [35].

2.2. Synthesis of $NaGdF_4$:Yb:Er nanoparticles

$NaGdF_4$:Yb:Er nanocrystals were synthesized using a thermal decomposition method. 1 mmol of Re $(CF_3COO)_3$ (Gd:Yb:Er = 78%:20%:2%), 2 mmol of CF_3COONa, and 20 ml solvent (10 ml OA/10 ml ODE) were added into a 100 ml three-necked flask simultaneously and degassed at 120 °C for 0.5 h under vacuum. In the presence of nitrogen, the mixture was rapidly heated to 320 °C and kept at this temperature for 1 h under vigorous magnetic stirring. After cooling down to the room temperature, the product was precipitated by addition of ethanol, separated by centrifugation, washed by cyclohexane, and then washed three times with ethanol. The yielded nanoparticles could be re-dispersed in various non-polar organic solvents.

2.3. Preparation of the UCNP@BSA

UCNPs were firstly coated with polyacrylic acid (PAA) following a literature methods [33]. The obtained PAA-modified UCNPs were soluble in water. To conjugate BSA to PAA-coated UCNPs, 100 mg BSA was mixed with 5 ml aqueous solution of PAA-coated UCNPs (2 mg/ml) and then added with 5 mg of 1-Ethyl-3- (3-dimethylaminopropyl) carbodimide (EDC). The solution was then stirred at room temperature for 2 h, added with another 5 mg of EDC, and then further stirred for 6 h at room temperature. The obtained UCNP@BSA nanoparticles were collected by centrifuging at 14,800 rpm for 5 min, with the precipitation re-dispersed in deionized water and stored at 4 °C.

2.4. Loading of RB and IR825 molecules on UCNP@BSA

In a typical experiment, loading of RB and IR825 into UCNP@BSA nanocomposite was accomplished by mixing 80 μM Rose Bengal and 0.4 mM IR825 with UCNP@BSA solution (0.2 mg/ml). The mixture was placed at 4 °C in the dark overnight. Excess RB and IR825 were removed by centrifugation and washing with deionized water for several times. The obtained UCNP@BSA-RB&; IR825 nanocomplex was stored at 4 °C in the dark. The UV–Vis–NIR absorbance spectra of UCNP@BSA-RB&; IR825 were measured by using UV765 (Shanghai Precision & Scientific Instrument Co. Ltd). The concentrations of Rose Bengal and IR825 loaded onto UCNPs were determined by their characteristic absorbance peak at 540 nm and 825 nm, respectively, after subtracting the corresponsive absorbance contribution from UCNP@BSA before drug loading at the same nanoparticle concentration.

The release of RB and IR825 from UCNP@BSA was studied by dispersing the sample under 37 °C in PBS and water for different periods of time. The released RB and IR825 from the nanocomplex were collected and determined by the UV–Vis–NIR spectroscopy.

2.5. Determination of singlet oxygen

Singlet oxygen sensor green (SOSG), which was highly sensitive to singlet oxygen, was employed here during the detection process. Different samples were mixed with 2.5 μM SOSG, and then irradiated by a 980-nm laser (0.5 W/cm^2) for different periods of time. The generation of singlet oxygen was determined by measuring recovered fluorescence of SOSG (excitation = 494 nm).

2.6. In vitro cell experiments

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. For confocal fluorescence imaging, 4T1 cells (1×10^5 cells) were cultured in 35 mm culture dishes containing different concentrations of UCNP@BSA-RB&; IR825 for 4 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) equipped with an external 980-nm excitation laser, to determine the cellular uptake of UCNP@BSA-RB& IR825.

The *in vitro* cytotoxicity was measured using a standard methyl thiazolyl tetrazolium (MTT, Sigma–Aldrich) assay. 4T1 cells were seeded into 96-well cell culture plate at 1×10^4/well until adherent and then incubated with various concentrations of UCNP@BSA, UCNP@BSA-RB, UCNP@BSA-IR825, and UCNP@BSA-RB& IR825 for 24 h. The standard MTT assay was carried out to determine the cell viabilities relative to the control untreated cells.

For *in vitro* PDT experiments, 4T1 cells (1×10^4 cells per well) seeded in 96-well plate were incubated with various concentrations of nanoparticles for 4 h, and then irradiated by the 980-nm laser at a power density of 0.4 W/cm^2 for 10 min, with 1-min interval for every 1 min of light exposure to avoid heating. Whereas for *in vitro* PTT experiments, cells after the nanoparticles incubation were exposed to the 808-nm laser at a power density of 0.5 W/cm^2 for 5 min. The cells were then incubated at 37 °C for additional 24 h before MTT assay to determine the cell viabilities relative to the control untreated cells.

For *in vitro* combined PDT/PTT experiment, 4T1 cells (1×10^4 cells per well) seeded in 96-well plate were incubated with 0.1 mg/ml UCNP@BSA-RB& IR825, and then irradiated by the 808-nm laser (0.5 W/cm^2, 5 min) and then 980-nm laser (0.4 W/cm^2, 1-min interval, 10 min). The cells were then re-incubated at 37 °C for additional 24 h before MTT assay to determine the relative cell viabilities.

2.7. Targeted cancer cell imaging

In order to increase the specificity, RGD was covalently linked to UCNP@BSA. Sulfosuccinimidyl 4-N-maleimidomethy cyclohexane-1-carboxylate (Sulfo-SMCC) was mixed with UCNP@BSA-RB& IR825 solutions at 1:10 M ratios at pH 7.4 for 2 h. Upon removal of excess reagents by centrifuging at 14,800 rpm for 5 min, the activated nanoparticles were reacted overnight with thiolated RGD at 1:5 M ratios at pH 7.4. The resulting UCNP@BSA-RGD nanoconjugates were collected by centrifugation and washed with PBS three times, re-dispersed in PBS, and stored at 4°C for further applications.

For confocal imaging, U87 cells over-expressing integrin $\alpha_v\beta_3$ receptor were seeded in 35 mm culture dishes and treated with 0.2 mg/ml of UCNP@BSA or UCNP@BSA-RGD for 2 h at 4°C. After washing with PBS (pH = 7.4) for three times, the cells were labeled with DAPI and then imaged by the confocal fluorescence microscope.

2.8. Tumor model

Nude 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 5×10^6 cells in ~30 μL of serum-free RMPI-1640 medium onto the back of each nude mouse. The mice were treated when the tumor volumes approached 50 mm^3.

2.9. In vivo PDT/PTT treatment

4T1 tumor-bearing mice were divided into 6 groups ($n = 5$ per group): (a) untreated; (b) irradiated by 980-nm + 808-nm laser; (c) intratumorally (i.t.) injected with 20 μl UCNP@BSA-RB&IR825; (d) i.t. injected with 20 μl UCNP@BSA-RB&IR825 and irradiated by the 980-nm laser; (e) i.t. injected with 20 μl UCNP@BSA-RB& IR825 and irradiated by the 808-nm laser; (f) i.t. injected with 20 μl UCNP@BSA-RB& IR825 and irradiated by both 980-nm and 808-nm lasers. The power density of 980 nm laser was 0.4 W/cm^2 (30 min, 1 min interval after each minute of irradiation), where as that of 808-nm laser was 0.5 W/cm^2 (5 min, continuous irradiation). An IR