NIR photoregulated theranostic system based on hexagonal-phase upconverting nanoparticles for tumor-targeted photodynamic therapy and fluorescence imaging

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Abstract

Although photodynamic therapy (PDT) is an effective, minimally-invasive therapeutic modality with advantages in highly localized and specific tumor treatments, large and deep-seated cancers within the body cannot be successfully treated due to low transparency to visible light. To improve the therapeutic efficiency of tumor treatment in deep tissue and reduce the side effects in normal tissue, this study developed a near-infrared (NIR)-triggered upconversion nanoparticle (UCNP)-based photosensitizer (PS) carrier as a novel theranostics system. The NaYF4:Yb/Er UCNPs were synthesized by a hydrothermal method, producing uniformly small size ([?]20 nm) nanoparticles and crystalline morphology of the hexagonal phase. These UCNPs were modified with folic acid-conjugated biocompatible block copolymers through a bidentate dihydrolipoic acid linker. The polymer modified hexagonal phase UCNPs (FA-PEAH-UCNPs) showed an improved dispersibility in the aqueous solution and strong NIR-to-vis upconversion fluorescence. The hydrophobic PS, pheophorbide a (Pha), was then conjugated to the stable vectors through a pH-sensitive linkage. Moreover, these UCNP-based Pha carriers containing tumor targeting folic acid ligands exhibited the significantly enhanced cellular uptake efficiency as well as PDT treatment efficiency. These results suggested that this system could extend the excitation wavelength of PDT to the NIR region and effectively improve therapeutic efficiency of PSs.

1. INTRODUCTION

Photodynamic therapy (PDT) is increasingly recognized as a promising treatment for a variety of cancers due to its low cost, highly localized and specific tumor treatments, fewer side effects as compared with radiation therapy and chemotherapy, and minimal trauma to organism tissue (Ackroyd, Kelty, Brown, & Reed, 2001; Dolmans, Fukumura, & Jain, 2003; Dougherty et al., 1998; Levy & Obochi, 1996). PDT is a powerful noninvasive therapeutic technique for a range of diseases including cancers, based on the photochemical reactions mediated by the interaction of photosensitizers (PSs) with specific light and molecular oxygen. Upon irradiation at the appropriate wavelength, the PSs become excited and transfer energy to oxygen in the surrounding tissue, generating highly reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$). The ROS moieties can react with biological molecules, resulting in an irreversible oxidative tissue damage and cell death (Allison et al., 2005; DeRosa & Crutchley, 2002; Dougherty, 1987; Wilson, 2002).

However, the principal problem limiting the use of many current PS in PDT is the low water solubility. These hydrophobic PSs could form aggregates in aqueous solution, which would reduce the$^1O_2$ quantum yield and affect the therapeutic efficiency of PDT. Additionally, because of their low water solubility, these PSs are difficult to prepare as pharmaceutical formulations for parenteral administration and cannot be directly injected intravenously (Konan, Gurny, & Allemann, 2002; B. H. Li et al., 2007). To overcome these limitations, various nanoscale drug carriers such as micelles (Woodburn & Kessel, 1994; G. D. Zhang et

al., 2003), liposomes (Ferro, Ricchelli, Mancini, Tognon, & Jori, 2006), dendrimers (Kim, Lee, Lee, Kim, & Kim, 2007), gold nanoparticles (Hone et al., 2002), mesoporous materials (Ideta et al., 2005) and carbon nanotubes (Liu et al., 2007; J. Wang, Liu, & Jan, 2004; Woolley, Guillemette, Cheung, Housman, & Lieber, 2000) have been explored as PS delivery systems in cancer therapy.

In addition, another main challenge for PDT is efficient treatment of cancers at a deep tissue level. However, the PSs used in conventional PDT are mostly excited by visible or even UV light, which has limited penetration depth due to the light absorption and scattering by biological tissues. PDT has been generally applicable to tumors on or just under the skin or on the lining of internal organs or cavities but does not produce effective therapeutic effects when treating large and deep-seated tumors (Detty, Gibson, & Wagner, 2004; C. Wang, Tao, Cheng, & Liu, 2011).

Near-infrared (NIR) light is referred to as the "optical window" of the biological tissues due to the minimal light absorption and scattering. Compared with the UV or visible light, NIR shows larger penetration distance in tissue, lower photodamage effects and higher signal-to-noise ratio (Du et al., 2010; Zhou et al., 2011). However, the current PSs for clinical usage, which can be efficiently activated by NIR light, remain rare.

Upconversion is an optical process that involves the conversion of lower-energy photons into higher-energy photons (Dong, Sun, & Yan, 2015; X. M. Li, Zhang, & Zhao, 2013; Zhou, Liu, & Li, 2012). Especially, lanthanide ion-doped upconversion nanoparticles (UCNPs) exhibit unique luminescent properties, including the ability to convert NIR long-wavelength excitation radiation into shorter visible wavelengths through a process known as photon upconversion. This process can further activate the PSs attached to nanoparticles to produce ROS. The advent of UCNPs would open a new pathway to full utilization of current and commercially available PSs upon NIR irradiation (Chatterjee, Gnanasammandhan, & Zhang, 2010; F. Wang, Banerjee, Liu, Chen, & Liu, 2010; P. Zhang, Steelant, Kumar, & Scholfield, 2007). In particular, UCNPs with a hexagonal phase have been demonstrated to be the best NIR-to-visible nanotransducers, which could provide the highest photon upconversion efficiency (Dong et al., 2015; X. M. Li et al., 2013; Zhou et al., 2012).

Recently, the UCNP-based PS delivery system for PDT has widely attracted interest from scientists, as it shows potential to overcome the above mentioned drawbacks of current PDT. However, there are still technical difficulties in the practical application of UCNP-based PS carriers. Also, the strategy of a UCNP-based theranostic system with a tumor-targeting ligand for selective PS delivery has not been reported much.

Therefore, we aimed to develop a NIR-regulated theranostic system based on hexagonal-phase UCNPs for tumor-targeted PDT and fluorescence imaging as shown in Figure 1. In this study, we optimized the hydrothermal synthesis procedure to produce $NaYF_4$:Yb/Er UCNPs with uniform size, hexagonal phase, and strong fluorescent intensity. In order to increase the aqueous solubility of UCNPs and introduce functional moieties into the surface of UCNPs for subsequent biological functionalization, folic acid-polyethylene glycol-poly(aspartic acid-hydrazone)-dihydrolipoic acid (FA-PEAH) polymer chains were conjugated. Then, a derivative of chlorophyll a, pheophorbide *a*(Pha), was conjugated to the side chain of FA-PEAH copolymer via an acid-labile hydrazone bond that is stable at physiological pH (7.0-7.4), but degraded at the lower pH (4.0-6.0) of the endosomal/lysosomal compartments. The size, size distribution, elemental composition, crystalline morphology, and luminescence properties of UCNPs were determined. To assess the potential of FA-PEAH-UCNPs-Pha as a NIR-triggered theranostic system, *in vitro* cellular localization and phototoxicity effects of UCNP-based nanocarriers were also investigated.

2. EXPERIMENTAL

2.1 Materials

Yttrium(III) chloride hexahydrate (YCl_3[?]$6H_2O$), ytterbium(Sh) chloride hexahydrate ($YbCl_3$[?]$6H_2O$), erbium(Sh) chloride hexahydrate ($ErCl_3$[?]$6H_2O$), ammonium fluoride (NH_4F), oleic acid, triphosgene, and 4-(dimethylamino) pyridine (DMAP) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA).

Sodium borohydride, N-hydroxysuccinimide (NHS), and N, N'-dicyclohexylarbodiimide (DCC) were purchased from Fluka (Buchs, Switzerland). 4-Hydroxy-2-butanone was purchased from TCI (Tokyo, Japan). α-Lipoic acid (LA), folic acid (FA), PEG-bis(amine) (molecular weight: 3.350 kDa), β-benzyl-L-aspartate (BLA), triethylamine (TEA), hydrazine monohydrate, sodium bicarbonate, and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), n-hexane, benzene, N, N-dimethylformamide (DMF), chloroform, diethyl ether, 1, 4-dioxane, methanol, dichloromethane (DCM), and acetic acid were obtained from Samchun Pure Chemical Co., Ltd. (Gyeonggi-do, Korea). Pheophorbide*a* (Pha) was purchased from Frontier Scientific, Inc. (Logan, UT, USA). Spectra/Por membranes were purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA, USA). All other chemicals were analytical grade.

2.2 Synthesis of tumor-targeted ligand and photosensitizer-conjugated UCNPs

2.2.1 Synthesis of hexagonal phase $NaYF_4$:Yb/Er UCNPs

YCl_3[?]$6H_2O$ (0.8 mmol), $YbCl_3$[?]$6H_2O$ (0.18 mmol), and $ErCl_3$[?]$6H_2O$ (0.02 mmol) were mixed with 25 ml oleic acid in a 250 ml flask. The solution was heated to 160 °C to form a homogeneous solution, and then cooled to room temperature. A 10 ml methanol solution containing NaOH (2.5 mmol) and NH_4F (4 mmol) was slowly added into the flask and stirred for 30 min. The solution was heated slowly to 100°C for 10 min to evaporate the methanol, and then heated to 300°C and maintained for 1 h under an N_2 atmosphere. After the solution was cooled naturally to room temperature, the nanocrystals were precipitated from the solution using ethanol and then washed three times with an ethanol/water (v/v=1:1) mixture (Z. Li & Zhang, 2008).

2.2.2 Surface modification of UCNPs

FA-conjugated block copolymer (FA-PEAH block copolymer), composed of tumor targeting ligand FA, PEG, poly(aspartate) and a dihydrolipoic acid end group, for surface modification of UCNPs was prepared as we reported previously (Zhao, Kim, Ahn, Kim, & Kim, 2013). The surface modification of UCNPs was performed by a ligand exchange method using the synthesized FA-PEAH block copolymers. The UCNPs (80 mg) were dispersed in 10 ml aqueous solution. The dispersion was stirred for 2 h while maintaining the pH at 4 by adding 0.1 M HCl solution to remove the oleate ligands. After the reaction, the aqueous solution was mixed with hexane to remove the oleic acid by extraction with hexane, and repeated three times. The combined hexane layers were re-extracted with water. In addition, the water layers were combined and re-extracted with hexane. The ligand-free UCNPs in the water dispersible fraction were collected by centrifugation after precipitation with cold acetone. The product was re-dispersed in acetone and the particles were collected by centrifugation. Finally, the particles were dispersed in water (20 ml) for future use. FA-PEAH (160 mg) was dissolved in 10 ml water, and the solution was added into ligand-free UCNP aqueous solution. The mixture was stirred at room temperature for 24 h, and then purified by dialysis against deionized water for 6 h. The resulting product was freeze-dried for further study (Bogdan, Vetrone, Ozin, & Capobianco, 2011; Naccache, Vetrone, Mahalingam, Cuccia, & Capobianco, 2009).

2.2.3 Preparation of Pha-conjugated UCNP nanocarriers (FA-PEAH-UCNPs-Pha)

To introduce the ketone groups to Pha molecules, Pha (0.176 mol) was dissolved completely in methanol (30 ml). EDC (0.53 mmol), DMAP (0.53 mmol), and 4-hydroxy-2-butanone (1.39 mmol) were added to the Pha solution. The reaction mixture was stirred at 400 rpm for 24 h at room temperature in the dark. Next, the solvent from the resulting mixture was removed in a vacuum oven, and the residue was washed with deionized water several times. The ketone group-modified Pha (Pha-HB) product was collected by centrifugation, and then freeze-dried.

Next, FA-PEAH-modified UCNPs (120 mg) were dissolved in DMSO (10 ml). The modified Pha (40 mg) dissolved in DCM (4 ml) was slowly added into the FA-PEAH-UCNPs solution. Subsequently, four drops of acetic acid were added into the mixture. The reaction mixture was stirred at 500 rpm for 24 h at room temperature in the dark. After the reaction, the DCM was removed under vacuum, and then DMSO was added into the residue to adjust the concentration of the mixture to about 3 mg/ml. After mixing, the solution

was dialyzed against $NaHCO_3$ (pH 8.0) solution for 1 day, and then against deionized water for another 12 h. The resulting product was centrifuged to remove unreacted Pha, and then the product was freeze-dried. In addition, an FA-unconjugated UCNP carrier sample (CH_3-PEAH-UCNPs-Pha) was synthesized using a similar method as a control group.

2.3 Characterization

The modification of copolymers was confirmed by 600 MHz ^{1}H NMR (AVANCE III 600, Bruker, Rheinstetten, Germany) using D_2O, and DMSO-d_6 as the solvent. Sizes and size distributions of UCNPs were determined by dynamic light scattering (DLS) (ELS-Z, OTSUKA, Japan) at 25oC using a He-Ne laser (633 nm) as a light source. The scattered light was measured at 90o and collected by an autocorrelator. The surface modification of UCNPs was determined by UV-visible spectrophotometry (UVmini-1240, Shimadzu, Japan), and energy dispersive spectroscopy (EDS) (Tecnai G^2 F30 TEM system). The morphologies of the nanoparticles were observed by field-emission transmission electron microscopy (FE-TEM) (Tecnai G^2 F30, FEI, Amsterdam, the Netherlands). The crystalline morphology of nanoparticles was also investigated by selected area electron diffraction (SAED) and X-ray Diffraction (XRD) (D8 DISCOVER, Bruker, Rheinstetten, Germany).

2.4 Cellular uptake of FA-PEAH-UCNPs-Pha

The MCF7 breast cancer cell is a FA receptor-overexpressing cell line (K. Li et al., 2011; Meier et al., 2010), used here for cellular uptake and phototoxicity tests. MCF7 cells (1x10^5 cells/well) were seeded onto 6-well plates and cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin-streptomycin at 37 in a humidified 5% CO_2-95% air atmosphere. After 24 h, the medium was replaced with 1.5 ml of fresh medium containing free Pha (10 μg/ml) and FA-PEAH-UCNPs-Pha (68 μg/ml, 10 μg/ml Pha equiv.), and then incubated for 4 h. The cells were then washed with PBS and harvested using 0.05% trypsin-EDTA. 4', 6-Diamidine-2-phenylindol (DAPI) and Alexa Fluor 488 phalloidin solutions were added to stain the cell nucleus and F-actin, respectively, at room temperature. All experiments were carried out in a dark room to prevent photodegradation of the probes. The cells were visualized using a confocal laser scanning microscope (LSM800, Carl-Zeiss, Germany).

2.5 *In vitro*phototoxi city assay of FA-PEAH-UCNPs-Pha

MCF7 cells (1×10^4 cells/well) were seeded onto 96-well plates in 200 μl RPMI 1640 and allowed to attach for 24 h. After cell attachment, the medium was replaced with 100 μl of fresh medium containing free Pha and FA/CH_3-PEAH-UCNPs-Pha under a series of concentrations (0, 5, 10, 20, and 30 μg/ml, Pha equiv.) with laser (980 nm) treatment at 0.1 mW/cm^2 for 5 min. Then, the irradiated cells were incubated at 37 ^{o}C for 24 h and cell viability was evaluated using a cell viability assay kit (CCK-8, DoGenBio, Korea). Untreated cells served as 100% viable cells. Data presented are averaged results of triplicate experiments. To determine the effect of laser exposure time on the phototoxicity, we also investigated the *in vitro* phototoxicity of free Pha, FA-PEAH-UCNPs-Pha, and CH_3-PEAH-UCNPs-Pha samples after laser (980 nm) radiation for 0, 0.5, 1, and 5 min at 0.2 mW/cm^2, the concentration of Pha was selected at 10 μg/ml (Pha equiv.). Dark-toxicity of the FA-PEAH-UCNPs-Pha was also evaluated by incubating for 4 h under 10 μg/ml (Pha equiv.) without laser irradiation.

3. RESULTS AND DISCUSSION

3.1 Synthesis and characterization of the UCNP-based nanocarrier

The synthetic scheme of the FA-PEAH-UCNPs-Pha nanocarrier is illustrated in Figure 2. Characterization of the biocompatible block copolymer, FA-PEAH, used for UCNPs modification was described in detail in our previous report[33]. As shown in Figure 3A, the synthesis of Pha-conjugated UCNP nanocarrier (FA-PEAH-UCNPs-Pha) were confirmed by the presence of the characteristic peaks of Pha at 8.9 ppm, 9.4 ppm, and 9.8 ppm, as well as characteristic peaks of FA at 7.2 ppm, PEG at 3.5 ppm, P(Asp) at 8.2 ppm, and DHLA at 1.2 ppm. In addition, the conjugation content of Pha to FA-PEAH-UCNPs was evaluated by ^{1}H NMR using the relative intensity ratio of the methylene protons of the PEG chain (-OCH_2CH_2-, 3.5 ppm) to

the methane protons of Pha (8.9 ppm, 9.4 ppm, and 9.8 ppm). The conjugation content of photosensitizer Pha in FA-PEAH-UCNPs-Pha carrier was determined as about 14.7%.

Furthermore, the formation and surface modification of lanthanide-doped $NaYF_4$:Yb/Er UCNPs were characterized by UV-visible spectroscopy and EDS measurements. Figure 3B showed the UV-visible absorption spectra of FA-PEAH-UCNPs and FA-PEAH-UCNPs-Pha. As shown in Figure 3B (b), the peak at about 279 nm was assigned to FA, while the peaks at about 401 nm, and 690 nm were attributed to Pha. This observation indicated that the Pha was introduced successfully to FA-PEAH polymer chain.

EDS was also employed to investigate the elemental composition of UCNPs before and after surface modification. As shown in Figure 3C, the characteristic peaks of F, Na, Yb, Y, Er, and C were observed in the free UCNPs sample. After FA-PEAH modification on the surface of UCNPs by a ligand cap exchange method, new N and S characteristic peaks belonging to the FA-PEAH copolymer appeared, and the relative intensity of the C peak increased significantly (Figure 3D). These results indicated that the $NaYF_4$:Yb/Er UCNPs were formed and the FA-PEAH layer was successfully immobilized onto the surface of UCNPs. After surface modification, the solubility of UCNPs improved significantly at the macroscopic level.

3.2 Morphology of UCNP-based nanocarriers

Size, size distribution, morphology and crystalline morphology of free UCNPs, FA-PEAH-UCNPs, and FA-PEAH-UCNPs-Pha were investigated by DLS, FE-TEM, SAED and XRD.

The morphologies of UCNPs, FA-PEAH-UCNPs and FA-PEAH-UCNPs-Pha were observed by FE-TEM. As shown in Figures 4A-C, These $NaYF_4$:Yb/Er nanocrystals were uniform submicron in size and monodisperse size distribution. The particle size was about 20 nm in dimeter with a hexagonal plate-like shape. The sizes of UCNPs were almost the same before and after modification in TEM images as shown in Figures 4A-C, while the sizes before and after modification were quite different in the DLS data (Figure 4D). Since TEM measurement is sensitive only to the electron dense metal particles, the size of all samples in the TEM images were almost the same and the polymers used for surface modification were not clearly observed. However, DLS measurement is sensitive to the hydrodynamic diameter of the whole nanocomposite. Thus, the DLS results of surface-modified UCNPs, FA-PEAH-UCNPs and FA-PEAH-UCNPs-Pha samples, exhibit a larger size than the TEM results.

Typical average particle size distributions measured by DLS for free UCNPs, FA-PEAH-UCNPs, and FA-PEAH-UCNPs-Pha are shown in Figure 4D as 996.0, 68.6, and 90.3 nm, respectively. Since free UCNPs were quite hydrophobic due to the hydrophobic oleate capping ligand before surface modification, the macroscopic aggregations were observed. Thus, DLS data of free UCNPs exhibited a much larger size compared with the surfaced-modified UCNP samples (FA-PEAH-UCNPs and FA-PEAH-UCNPs-Pha). However, after surface modification of the hydrophilic FA-PEAH polymer instead of the hydrophobic oleate ligand, the dispersity and solubility in aqueous solution significantly improved. As shown in Figure 4D, the size of the nanoparticles significantly decreased (about 68.6 nm for FA-PEAH-UCNPs and 90.3 nm for FA-PEAH-UCNPs-Pha), and the size distribution maintained a narrow monodisperse unimodal pattern.

In order to evaluate the deep-penetration PDT application, hexagonal-phase UCNPs are the best choice, because hexagonal-phase $NaYF_4$:Yb/Er UCNPs usually produce a bright green emission (around 550 nm) along with a weak dark red emission (around 660 nm) under 980 nm NIR irradiation(F. Wang & Liu, 2009). Also, it has been reported that hexagonal-phase $NaYF_4$($\beta NaYF_4$) crystals are the most efficient host materials for upconverting lanthanide ions due to the low phonon energy of the crystal lattice. The crystalline morphology of the synthesized $NaYF_4$:Yb/Er UCNPs was investigated by the SAED pattern, as shown in Figure 4E. The SAED pattern of the UCNPs was shown as spotty polycrystalline diffraction rings, which can be indexed to the (100), (110), (101), (110), (200), (111), (201), (210), (002), (300), (211), and (321) planes of hexagonal $NaYF_4$lattice. We also employed XRD to further confirm the crystalline morphology of $NaYF_4$:Yb/Er UCNPs. In Figure 5, the peak positions and intensities of the free UCNPs agree well with the standard pattern of hexagonal phase $NaYF_4$ crystal (Figure 5A; , JCPDS 16-0334). These results indicated

that the synthesized $NaYF_4$:Yb/Er UCNPs have the hexagonal β -phase. Additionally, in Figure 5B, the peak positions between 15 and 30 degrees were well matched with the standard pattern of PEG (V, JCPDS 49-2095). It also could be evidence that the FA-PEAH layer was successfully immobilized onto the surface of UCNPs.

3.3 Luminescence properties of UCNPs

The upconversion fluorescence spectra of surface modified $NaYF_4$:Yb/Er UCNPs (FA-PEAH-UCNPs) in aqueous solution excited with a 980 nm laser at room temperature are shown in Figure 6. The FA-PEAH-UCNPs sample shows three district Er^{3+}emission bands. The sharp green emissions between 510 and 530 nm and between 530 and 570 nm were assigned to the$^2H_{11/2}$ -$^4I_{15/2}$ and$^4S_{3/2}$ -$^4I_{15/2}$ transitions, respectively. A red emission was also observed between 645 and 680 nm corresponding to the $^4F_{9/2}$ -$^4I_{15/2}$ transition. The inset in Figure 6 exhibits photographs of free UCNPs and surface modified $NaYF_4$:Yb/Er UCNPs in aqueous solution under 980 nm laser irradiation. The free UCNPs and FA-PEAH-UCNPs sample show a yellowish green color upon excitation by a 980 nm laser. The red emission band of UCNPs between 645 and 680 nm exhibits a good match for the main absorption band of Pha between 645 and 735 nm. This result indicates that the photosensitizer Pha molecules could be activated by luminescence intensity of $NaYF_4$:Yb/Er UCNPs upon 980 nm laser irradiation.

3.4 Cellular localization of UCNP-based nanocarrier in tumor cells

The efficiency of PDT treatment is highly dependent on PS cellular uptake and accumulation in malignant tissues (Nawalany et al., 2009). The fate of the UCNP-based carrier in tumor cells were investigated using confocal laser scanning microscopy. As shown in Figure 7, the confocal microscopy assay was based on the red autofluorescence of Pha, and the blue fluorescence from DAPI bound to the nucleus, and F-actin in the cytoplasm stained by Alexa Fluor 488 phalloidin.

For MCF7 cells treated with free Pha, the red fluorescence signal from Pha detected in MCF7 cells was quite weak (Figure 7B). On the other hand, UCNP-based Pha carriers, FA-PEAH-UCNPs-Pha and CH_3-PEAH-UCNPs-Pha, showed higher red fluorescence Pha signal compared with the free Pha sample. Especially, after FA-PEAH-UCNPs-Pha treatment, MCF7 cells produced strong red fluorescence from Pha around the nucleus and at the inner part of the cells, indicating that Pha molecules were effectively internalized into the MCF7 cells (Figure 7D). The results indicate that the UCNP-based Pha carrier improved the Pha water solubility, and FA ligands could effectively enhance the cellular uptake of Pha molecules into MCF7 cells by an active targeting effect. These strong fluorescence signals from Pha could be also used to clearly understand the dynamics of signal transduction in the intracellular networks and in diagnostics.

3.5 *In Vitro* phototoxicity test of UCNP-based nanocarriers

To determine the PDT efficacy of the UCNP-based Pha nanocarrier, the*in vitro* cytotoxicity of free Pha, FA-PEAH-UCNPs-Pha, and CH_3-PEAH-UCNPs-Pha was measured. For the phototoxicity test, we investigated the phototoxicity against MCF7 cells using various concentrations of Pha (0, 5, 10, 20, and 30 μg/ml) and laser exposure times (0, 0.5, 1, and 5 min). After 980 nm laser radiation for 5 min at 0.1 mW/cm^2, FA-PEAH-UCNPs-Pha and CH_3-PEAH-UCNPs-Pha exhibited significantly enhanced phototoxicity compared to free Pha as shown in Figure 8A. As the Pha concentration increased, the cell viability gradually decreased. Notably, the viability of MCF7 cells treated with FA-PEAH-UCNPs-Pha sample decreased to nearly 25% after a 5 min treatment at a concentration of 30 μg/ml. In order to determine the effect of laser exposure time on the phototoxicity, we also determined the *in vitro* phototoxicity of free Pha, FA-PEAH-UCNPs-Pha, and CH_3-PEAH-UCNPs-Pha after 980 nm laser radiation for 0, 0.5, 1 and 5 min at 0.2 mW/cm^2 (10 μg/ml, Pha equiv.). Under the dark condition, free Pha, FA-PEAH-UCNPs-Pha, and CH_3-PEAH-UCNPs-Pha exhibited more than 90% cell viability and no significant dark toxicity as shown in Figure 8B (0 min of laser exposure time, 10 μg/ml Pha concentration). However, the cell viability significantly decreased as the laser exposure time increased (Figure 8B).

The PDT efficiency of FA-PEAH-UCNPs-Pha was obviously higher than free Pha and CH_3-PEAH-UCNPs-

Pha. These results are probably due to the increased solubility of hydrophobic Pha molecules in aqueous environments by loading into the block copolymer chain-immobilized UCNP carriers, resulting enhanced $^1O_2$quantum yield[37] of Pha. In addition, FA-conjugated FA-PEAH-UCNPs-Pha could exhibit the higher phototoxicity than free Pha and CH_3-PEAH-UCNPs-Pha since the cellular uptake was improved by the FA receptor-medicated endocytosis process.

4. CONCLUSIONS

In order to demonstrate the UCNP-based cancer therapies, particularly NIR-light induced photodynamic therapy, we have designed the NIR light-triggered theranostic system based on hexagonal-phase $NaYF_4$:Yb/Er UCNPs for efficient PDT with enhanced deep tissue penetration ability and fluorescence imaging. Hexagonal-phase $NaYF_4$:Yb/Er UCNPs were synthesized by a hydrothermal method and the nanoparticles were monodisperse with a uniform size of about 20 nm. The crystalline morphology of the synthesized $NaYF_4$:Yb/Er UCNPs showed a thermodynamically stable hexagonal β-phase. Since the UCNPs have no intrinsic aqueous solubility and lack functional moieties for subsequent biological functionalization, these UCNPs were modified with FA-conjugated biocompatible block copolymers through a bidentate dihydrolipoic acid linker. The FA-PEAH copolymer-modified UCNPs (FA-PEAH-UCNPs) showed improved solubility and dispersibility in aqueous solution. Then, the hydrophobic PS, Pha, was conjugated to the stable vectors through a pH-sensitive linkage. These water dispersible UCNPs have a much stronger luminescence property compared with hydrophobic UCNPs. The upconversion fluorescence spectra of FA-PEAH-UCNPs excited with a 980 nm laser showed sharp green emissions between 510 and 530 nm and between 530 and 570 nm as well as a red emission between 645 and 680 nm. These FA-PEAH-UCNPs-Pha that produce high energy visible photons from low energy radiation in the NIR region could be very promising materials for bioimaging and PDT. The advantage of NIR radiation use is less harmful to cells, minimizes auto-fluorescence from biological tissues, and penetrates tissues to a greater extent. Due to the active tumor targeting FA ligand conjugation, the cellular uptake and phototoxicity against MCF7 cells of FA-PEAH-UCNPs-Pha were significantly enhanced compared with free Pha and FA-ligand unconjugated CH_3-PEAH-UCNPs-Pha. These UCNP-based Pha nanocarriers containing tumor targeting FA ligands and pH-sensitive cleavage sites could improve the solubility of Pha and increase $^1O_2$ quantum yield in the weakly acidic conditions of tumor tissue, as well as promote PDT treatment efficiency. In addition, this nanocarrier could be triggered by NIR which has deep tissue penetration. These results suggest that the FA-PEAH-UCNPs-Pha system has potential use as an effective PS delivery system for tumor PDT applications in deep tissue.

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Figure Legends

Figure 1