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Supporting Information

Photosensitizer conjugated iron oxide nanoparticles for simultaneous *in vitro* magneto-fluorescent imaging guided photodynamic therapy

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Materials & Methods

1. Materials

N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), *N*-Hydroxysuccinimide (NHS), Fe (II) and Fe (III) chlorides, Tetramethyl ammonium hydroxide (TMAOH), 3-Aminopropyl) triethoxysilane (APTES), MES buffer and MTT were purchased from Sigma-Aldrich (MO, USA). Heparin was purchased from Mediplex Corporation (Korea). DMA were purchased from Fluka Analytical (MO, USA). Pheophorbide A was purchased from Frontier Inc (Tokyo, Japan).

2. Synthesis of Iron oxide (Fe_3O_4) nanoparticles

Fe_3O_4 nanoparticles were synthesized by chemical co-precipitation of Fe (II) and Fe (III) chlorides and NaOH as the reductant following a reported method with minor modification.[1] Briefly, 10 mM of $FeCl_3 \cdot 6H_2O$ and 5 mM of $FeCl_2 \cdot 4H_2O$ were dissolved in 25 mL of deionized water containing 0.01 M of HCl. A solution of NaOH (1.5 M, 250 mL) was added drop wise to the mixture under vigorous stirring. Black Fe_3O_4 nanoparticles were formed immediately and were isolated using a permanent magnet, followed by washing twice with 250 mL of deionized water and twice with 0.1 M TMAOH. Fe_3O_4 nanoparticles were collected by centrifugation at 12,000 rpm for 15 min. To obtain oxidized Fe_3O_4 nanoparticles, the precipitates were washed with 0.1 M HNO_3 and isolated by centrifugation. Subsequently, the particles were dissolved in 0.01 M HNO_3 and heated while stirring at 95˚C until the color of the solution changed brown. After the solution was cooled, the oxidized Fe_3O_4 nanoparticles were isolated, washed several times with deionized water, and finally suspended in 100 mL of 0.1 M TMAOH.

3. Surface modification of Fe_3O_4 nanoparticles

APTES was used to modify the surface of Fe_3O_4 nanoparticles with silica by following the literature with minor modifications.[2] In brief, 100 mg of Fe_3O_4 nanoparticles were washed once with methanol (20 mL), then with a mixture of methanol and toluene (20 mL; 1:1, v/v), and finally with toluene alone (20 mL). Later Fe_3O_4 nanoparticles were dispersed in toluene (20 mL), and 0.1 mL of APTES [5 mM in a methanol/toluene (1:1, v/v) mixture] was added to the Fe_3O_4 nanoparticles suspension. The suspension was refluxed at 110 °C for 24 h under a N_2 flow and vigorous stirring. The modified particles were magnetically collected, washed with methanol three times, and dried in a vacuum. APTES coating on the surface of Fe_3O_4 nanoparticles (APTES@Fe_3O_4) were also confirmed by XRD and XPS (Thermo Fisher Scientific, USA).

4. Aminated pheophorbide A

Synthesis of aminated pheophorbide A (Aminated PheoA*)* was based on our previous report.[3] PheoA (150 mg) was activated with EDC (61.6 mg) and NHS (61.6 mg) in 10 mL of dimethylformamide (DMF) at room temperature for 12 h. Later ethylenediamine (36 µL) and pyridine (20 µL) was added and incubated for 12 h at room temperature, followed by dialysis for 1 day against deionized water to remove by products, organic solvent and unreacted compounds. The dark green powder was obtained after ultracentrifugation and freeze-drying.

5. Aminated pheophorbide A conjugated heparin

Aminated PheoA conjugated heparin (PheoA-Hep) was prepared by a method proposed by Li *et al.*[4] Briefly 50 mg of Aminated PheoA and 160 mg of heparin were dissolved in formamide (5 mL) and DMF (5 mL), respectively. Two solutions were mixed under vigorous stirring. Later 90 mg of EDC were added to the mixture and the reaction was carried out at room temperature for 36 h under nitrogen atmosphere. After the reaction, the mixture was

dialyzed and freeze-dried flowed by washing three times with methanol to obtain a purified product.

6. PheoA-Hep conjugated with APTES@Fe_3O_4 nanoparticles

Amide bond assisted conjugation of PheoA-Hep and APTES@Fe_3O_4 nanoparticles were synthesized by dissolving 50 mg of APTES@Fe_3O_4 nanoparticles in 10 mL of MES buffer (0.1 M, pH - 6.5) containing 50 mg PheoA-Hep. Later, EDC (40 mM) and NHS (100 mM) were added to this solution. The mixture was sonicated for 10 min at 4 °C followed by shaking for 24 h at room temperature. The final conjugated mixture was collected under an external magnetic field and washed with a MES buffer and ethanol.[2]

7. Characterization of PheoA-Hep conjugated with APTES@Fe_3O_4 nanoparticles

The morphology and size of the aqueous dispersion of the PheoA-Hep conjugated with APTES@Fe_3O_4 (PheoA-Hep-Fe_3O_4) nanoparticles were characterized using Field emission scanning electron micrograph (FE-SEM) (JSM-7000F, JEOL, Japan), operating at an accelerating voltage of 200 kV. The X-ray diffraction pattern of Fe_3O_4 nanoparticles and PheoA-Hep-Fe_3O_4 nanoparticle were obtained using Rigaku D/max-2550 PC with monochromated CuK*a* radiation and XPS (Thermo Fisher Scientific, US). UV–vis spectra were measured at 20 °C with UV-vis spectroscopy (Mecasys Co. Ltd., Korea) equipped with a 10-mm quartz cell with a path length of 1 cm. Fluorescence spectra were recorded on a PL spectroscopy (Sinco, Korea). The fourier transform infrared spectra were acquired using FT-IR Spectrometer (Bruker, Germany) in the wavenumber range of 4000 – 400 cm^{-1}. The chemical structure of PheoA, Aminated PheoA and PheoA-Hep were also confirmed by ^{1}H-NMR (Bruker, Germany). The surface charges of PheoA-Hep-Fe_3O_4 nanoparticles were performed on Zetasizer ELS Z (Otsuka Electronics, South Korea). Thermal-gravimetric

analysis (TGA) curves were recorded on a TA-Q50 thermo-gravimetric analyzer (TGA) (TA, DE). The standard curve was established in one range of drug concentration. The loading Efficiency was evaluated by TGA. Coupling efficiency of PheoA-Hep-Fe_3O_4 nanoparticle was carried out by TNBSA assay.

8. Single oxygen detection

Due to high reactivity, singlet oxygen is thought to have a very short lifetime and hence it is difficult to be detected. A commercially available reagent DMA, which is highly selective for singlet oxygen, was applied to a range of biological systems that are known to generate singlet oxygen. To evaluate the singlet oxygen generation of PheoA-Hep-Fe_3O_4 nanoparticles in aqueous solution, 9,10-dimethylanthracene (DMA) was added to evaluate the $^1O_2$ generated. Solutions of PheoA-Hep-Fe_3O_4 nanoparticles containing DMA (1.184×10^{-2} mM) were irradiated at RT using a 670 nm laser source (B&W, USA) at a light intensity of 4 mW/cm^2. The decrease in fluorescence intensity of DMA (emission from 380 to 550 nm, with excitation at 360 nm) as a result of $^1O_2$ generation was monitored using a fluorescence spectrometer.[4]

9. Cellular uptake study

KB cells (5×10^4 cells/mL) were seeded in 8-well plates and incubated overnight at 37 °C in a humidified 5% CO_2 atmosphere. After 4 h of incubation with 50 μg/mL PheoA of PheoA-Hep-Fe_3O_4 nanoparticles, the cells were rinsed with PBS and observed under fluorescence microscopy.[5]

10. *In vitro* PDT effect

Different 96-well plates were divided into two groups: control and experimental group. KB cells (5×10^4 cells/mL) were seeded, exposed identically to the plates prepared for the cytotoxicity assessment. The cells were then treated with PheoA-Hep-Fe_3O_4 nanoparticles at PheoA concentrations of (0-50) μg/mL. For laser dependent assay, cells were then treated with PheoA-Hep-Fe_3O_4 nanoparticles at PheoA concentrations of 5 μg/mL. Then, the cells of experimental group were rinsed with PBS and immersed in 100 μL of fresh culture medium before being irradiated using a 670 nm laser with energy density of (0-4) mW/cm^2 (laser dependent assay) and 4 mW/cm^2 (conc. dependent assay) for 10 min . After irradiation, cells were incubated another 24 h in a 5% CO_2, 95% air humidified incubator at 37 °C. Dark control group keeps identical to experimental group without irradiation. PDT effect assay was also determined by MTT assay.[6]

11. *In vitro* MRI of PheoA-Hep-Fe_3O_4

A series of aqueous solutions of PheoA-Hep-Fe_3O_4 nanoparticles (with Fe from 0 to 0.25 mM) was prepared and imaged in a 24-well plate on a 1.5 T clinical MRI instrument (GE Signa Excite Twin-Speed, GE Healthcare, Milwaukee, USA). A T2-weighted conventional spin echo sequence was set as follows: TR/TE =2400 ms per various echo times (including 12, 16, 20, 24, 32, 36, 40, 48, 60, 64, 80, 120, and 160 ms), Field of view = 14 cm, slice thickness = 2 mm, inter slice gap = 0.1 mm and matrix =256 ×192.[5]

12. Statistical Analysis

Significant differences were assessed with a one-way analysis of variance (ANOVA) (Origin Pro 8.0), and the data were presented as the means ± S.E

Fig. S1. X-ray Diffraction patterns of Fe_3O_4 and APTES@Fe_3O_4 (Inset: magnetivity of APTES@Fe_3O_4 nanoparticles).

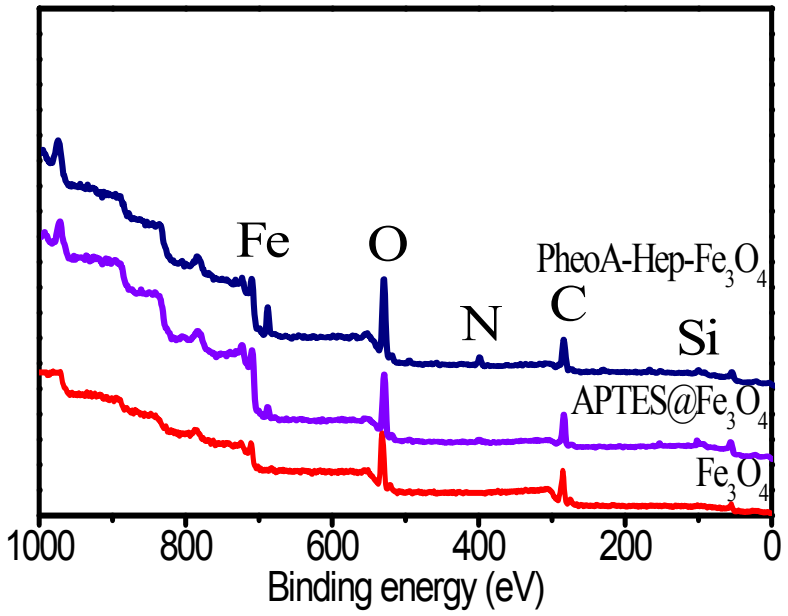

Fig. S2. XPS wide-scan spectra of Fe_3O_4, APTES@Fe_3O_4 and PheoA-Hep-Fe_3O_4.

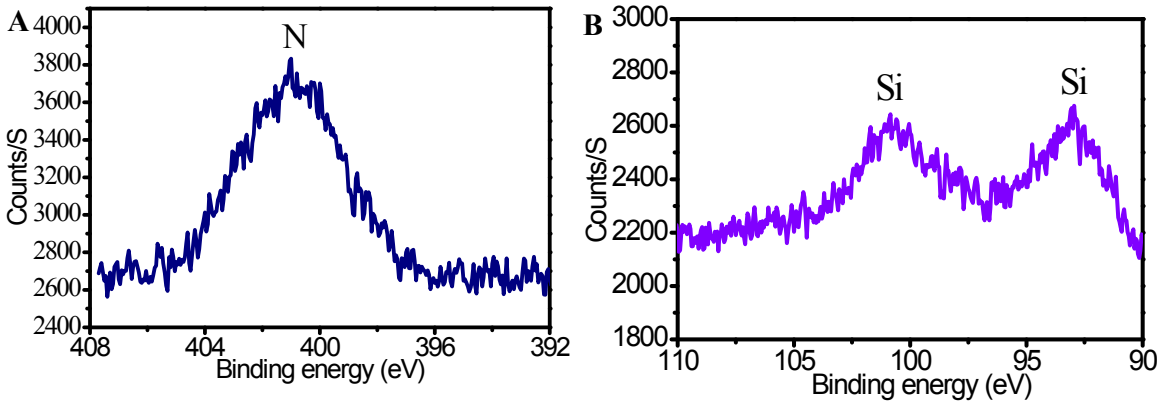

Fig. S3. High-resolution Si spectra of APTES@Fe_3O_4 (A) and N spectra of PheoA-Hep-Fe_3O_4 (B).

Fig. S4. ^{1}H-NMR spectra of PheoA, Aminated PheoA and Aminated PheoA conjugated Heparin.

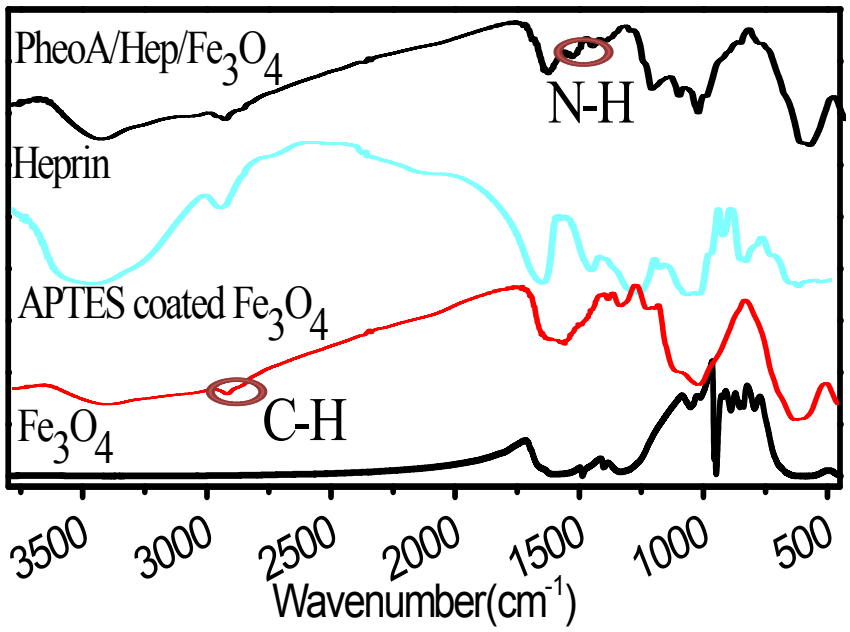

Fig. S5. FT-IR spectra of Fe_3O_4, APTES@Fe_3O_4, Heparin and PheoA-Hep-Fe_3O_4 nanoparticles.

Table S1. Number of amine detection by TNBSA assay

Name of Product	Number of amine	Number of PheoA-Hep
Fe_3O_4	0	0
APTES@Fe_3O_4	69	0
PheoA-Hep-Fe_3O_4	39	30

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