Supporting Information

# A Strategy for Effective Radioprotection by Chitosan-Based Long-Circulating Nanocarrier

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## 1. General

### Materials and animal

Chitosan (decetylation degree = 95.2%, Mn = 50,000 g/mol) was kindly provided by Golden-Shell Biochemical Co. Ltd. (Zhejiang, China). Sulfobetaine methacrylate (SBMA), Ferulic Acid (FA), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazoliumbromide (MTT) were purchased from Sigma-Aldrich (Shanghai, China). S, S'-bis (R,R'-dimethyl-R''-acetic acid) trithiocarbonate (BDACT) was synthesized for graft polymerization according to a literature method (Figure S8) [1]. Cy5.5 was purchased from Azco biotech co. (USA). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were obtained from Hyclone (Logan, Utah). Human dermal fibroblasts (HDF) were purchased from the Hygia biotech co. (Suzhou, China). All other chemical agents were used as received. Formula of PBS buffer solution: NaCl 8.0 g, KCl 0.20 g, Trisodium Citrate 1.0 g, $NaHCO_3$ 1.0 g and $NaH_2PO_4.2H_2O$ 0.057 g, dissolved in water (1 L).

Ten-week-old male SD rats and eight-week-old male C57BL/6 mice (Silaike Experimental Animal, Shanghai, China) were maintained in a laboratory animal facility with temperature and relative humidity maintained at 23 ± 2ºC and 50 ± 20%, respectively. All mice were given a standard chow diet and water ad libitum. All experimental procedures were in accordance with the guidelines provided by the Animal Ethical Committee of Soochow University.

**Characterization methods.** $^1$H nuclear magnetic resonance ($^1$H NMR) spectra were taken by a Varian INVOA-400 instrument operated at 400 MHz. Fourier transform infrared (FT-IR) spectra were obtained on a Varian-1000 spectrometer; the samples were ground with KBr crystals, and the mixture was then pressed into a pellet for IR measurement. Field-emitting scanning electron microscopy (SEM) images were obtained by a Hitachi S-4700 microscope working at an accelerating voltage (15 kV). Malvern Zetasizer with irradiation (He-Ne laser, 632.8 nm) was used to determine Zeta potential and Z-average size distribution of the nanoparticles. Absorbance in MTT was measured at 570 nm with a Synergy 2 microplate reader (BioTek, USA). The fluorescence intensities of Cy5.5 in serum were measured by a Synergy NEO microplate reader (BioTek, Vermont) with excitation (675 nm) and emission (705 nm). The fluorescence images of Cy5.5 in the organs were taken by FX Pro (Kodak, USA) in-vivo imaging system. Blood samples were collected and analyzed by CELL-DYN 3700 blood cell analyzer (Abbott, Chicago).

**2. Synthesis of BDACT for controlled polymerization**

BDACT was synthesized according to the related reference[1]. Specifically, carbon disulfide (11 mL, 0.18 mol), chloroform (36 mL, 0.45 mol), acetone (33 mL, 0.45 mol), and tetrabutylammonium bromide (1.16 g, 3.6 mmol) were mixed with 60 mL of petroleum ether in a 1L round-bottomed flask cooled with ice-water bath under argon. Then 50% sodium hydroxide (1.26 mol) was added dropwise. The reaction mixture was further stirred for 10 h, 450 mL of water was then added to dissolve the solid, followed by 60 mL of concentrated HCl to acidify the aqueous layer. The crude product was filtered and rinsed thoroughly with water, and then it was further purified by recrystallization in toluene/acetone (4/1). The structure of BDACT was confirmed by $^{1}$H NMR. (Figure S8).

**3. Synthesis of CS-*g*-PSBMA copolymer**

A typical graft polymerization is described below [2]: Chitosan (0.12 g, 0.015 g/mL), BDACT (0.0212 g, 0.08 mmol), and SBMA (0.12 g, 0.43 mmol) were dissolved with 8 mL mixture of 1% HCl aqueous solution and acetone (v/v = 7/3) in a 10 mL ampoule. After the contents were purged with argon for 20 min to eliminate the oxygen, the ampoules were flame-sealed. Then the ampoules were placed in an insulated room with a $^{60}$Co source at the dose rate of 10 Gy/min for 16.7 h. The reaction mixture was dialyzed against distilled water with a membrane (MWCO = 25,000 Da) for 72 h to remove un-reacted SBMA, PSBMA homopolymers, HCl and acetone. Finally, the dialyzate was lyophilized to afford chitosan graft copolymer CS-*g*-PSBMA. The graft content was determined by gravimetry.

The trithio group of the chain transfer agent BDATC was stable in the presence of 1% HCl. The intermediate macro-initiator structure of CS-BDATC was characterized in the related reference. [2]

**4. Loading FA onto CS-*g*-PSBMA nanoparticles**

FA (40 mg) was dissolved in 1.5 mL of ethanol, and then was slowly added into CS-*g*-PSBMA aqueous solution (3.33 mg/mL, 15 mL) under strong stirring. The mixture solution was purged using nitrogen gas to eliminate ethanol and disperse FA with CS-*g*-PSBMA overnight. The suspension was then centrifuged at 8000 r/min for 20 min to remove the unpackaged FA. The supernatant was lyophilized to afford CS(FA)-*g*-PSBMA nanoparticles.

To test the loading efficiency, 5.0 mg of CS(FA)-*g*-PSBMA nanoparticles were redispersed in

ethanol (3.0 mL) under ultrasonication for 2 h, and then was filtered under reduced pressure to remove the CS-*g*-PSBMA carriers by an organic membrane (pore size: 0.22 μm). The concentration of FA in ethanol was then determined according to the standard curve (Figure S9) by UV/visible spectroscopy at 322.5 nm. On the basis of the calculation of weight of FA ($W_{FA}$) and CS-*g*-PSBMA ($W_{CS\text{-}g\text{-}PSBMA}$), FA loading efficiency (LE, in wt %) was estimated as LE = $W_{FA}$/ $W_{CS\text{-}g\text{-}PSBMA}$ × 100%.

**5. In vitro drug stability of CS(FA)-*g*-PSBMA nanoparticles**

Release behavior of FA from CS(FA)-*g*-PSBMA nanoparticles was investigated at pH 7.4 (similar pH of physiological blood). CS(FA)-*g*-PSBMA nanoparticles were dispersed in PBS (pH 7.4, 5 mL) and transferred into a dialysis bag (MWCO = 3,500 Da). The dialysis bag was then immersed in 200 mL of PBS at 25 °C and 37 °C. The release medium was continuously agitated with a stirrer. The amount of released FA in PBS was then determined according to the standard curve (Figure S10) by UV/visible spectroscopy at 309 nm.

**6. Antioxidant activity of CS(FA)-*g*-PSBMA nanoparticles by DPPH assay**

The antioxidant activity of CS(FA)-*g*-PSBMA nanoparticles was evaluated by DPPH assay according to the literature method [3]. Chitosan (0.71 mg), FA (0.29 mg) and CS(FA)-*g*-PSBMA nanoparticles (1 mg) were added into ethanol solution of the stable DPPH radical (100 μM, 3 mL), respectively, and then incubated in the dark for 8 h. The absorbance of the DPPH solution was measured at 517 nm. The radical scavenging activity was defined as a decrease in the absorbance of DPPH, and was calculated using the following formula:

DPPH decoloration (%) = $(A_{control} - A_{sample})/A_{control} \times 100$

Where $A_{control}$ is the absorbance of the supernatant in a control tube, and $A_{sample}$ is the absorbance of the supernatant in a sample tube.

**7. Radioprotective effect of CS(FA)-*g*-PSBMA by MTT assay**

The radioprotective effect of CS(FA)-*g*-PSBMA was investigated in HDF using MTT assay in comparison with FA and CS-*g*-PSBMA. The cell were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin and 100 units/ml penicillin at 37 °C under a humidified, 5% $CO_2$ atmosphere. Cells were seeded at a density of 5000 cells/well in 96-well plates. FA stocking solution was prepared in DMEM at 1 mg/mL by 30 min of sonication. CS-*g*-PSBMA and CS(FA)-*g*-PSBMA solutions were obtained by the same

method. The cells were incubated with various concentrations of CS-*g*-PSBMA，FA and CS(FA)-*g*-PSBMA solution for 12 h, and then exposed to 4 Gy γ-ray irradiation with a $^{60}$Co source. After 24 h post-irradiation, cells were incubated with 50 μg/100 μL MTT solution at 37°C for 4 h, and 100 μL DMSO was then added to dissolve the formazan crystals. The absorbance was measured at 570 nm using a spectrophotometer. The microscopic images of the cells were taken by an inverted microscope (Olympus, Tokyo, Japan).

**8. Cy5.5-labeled CS(FA)-*g*-PSBMA and CS-*g*-PSBMA nanoparticles. [4]**

To label Cy5.5 to CS(FA)-*g*-PSBMA nanoparticles or CS-*g*-PSBMA nanoparticles, 1 wt % hydroxysuccinimide ester of Cy5.5 was dissolved in DMSO and mixed with CS(FA)-*g*-PSBMA nanoparticles or CS-*g*-PSBMA nanoparticles solution. The reaction was performed at room temperature in the dark for 6 h. Byproducts and unreacted Cy5.5 molecules were removed over a period of two days by dialysis (molecular weight = 14 KDa) against distilled water, and the resulting product was lyophilized.

**9. Pharmacokinetics and bioavailability of CS(FA)-*g*-PSBMA nanoparticles in SD rats**

Pharmacokinetics and tissue distribution was investigated as described below: Cy5.5-labled CS-*g*-PSBMA (17 mg/kg in 500 μL of saline) and Cy5.5-labled CS(FA)-*g*-PSBMA (LE = 41.6 %, 5 mg/kg of FA in 500 μL of saline) were intravenously administrated to SD rats (n = 3 for each group). Blood samples were collected through tail vein at different time points. Then the blood samples were centrifuged at 5000 rpm for 10 min to obtain serum. The fluorescence intensities of Cy5.5 in serum were measured by a Synergy NEO microplate reader (Bio Tek, Winooski, VT) with excitation at 675 nm and emission at 705 nm. For biodistribution study, SD rats were sacrificed and the organs (including liver, heart, kidney and spleen) were harvested, and the fluorescence of Cy5.5 in the organs was imaged by Kodak in-vivo imaging system.

**10. In vivo therapeutic efficacy of CS(FA)-*g*-PSBMA nanoparticles in mice**

FA (6.25 mg/kg in 100 μL of saline) and CS-*g*-PSBMA (15 mg/kg in 100 μL of saline) and CS(FA)-*g*-PSBMA (41.57 wt.%, 6.25 mg/kg of FA in 100 μL of saline) were intravenously administrated to C57BL/6 mice (n = 3-5 for each group). One hour after injection, mice were subjected to 4 Gy whole-body irradiation with a $^{60}$Co source. After 3 days, blood samples were collected and analyzed by CELL-DYN 3700 blood cell analyzer (Abbott, Chicago).

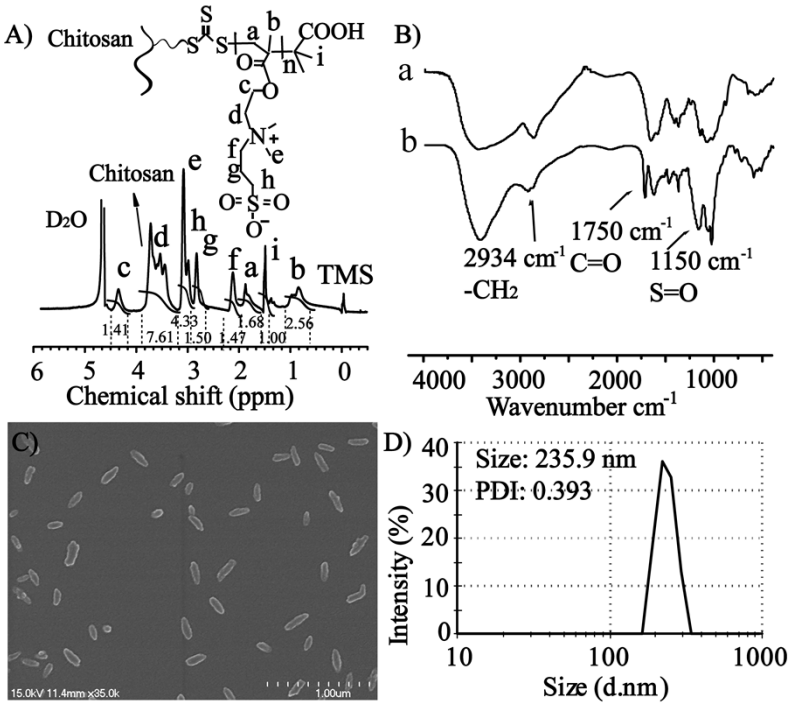


**Figure S1.** (A) $^{1}$H NMR spectrum (400M, $CF_3COOD/D_2O$ (v/v = 1/9)) of CS-*g*-PSBMA (G % = 53.17 %). (B) FT-IR spectra of (a) chitosan and (b) CS-*g*-PSBMA. (C) SEM image (scale bar: 1.00 μm) and (D) particle size distribution of CS-*g*-PSBMA (G% = 53.17 %) in distilled water. (The molecular weight of grafted PSBMA could be calculated as 1600 g/mol from the equation: $M_{n,NMR}$ = 293 * $I_g$ * 6 / ($I_i$ * 2) + 282, where $I_g$ and $I_i$ are the integral values of the peaks which correspond to -$CH_2$- in PSBMA and -$CH_3$ in BDACT, respectively; 293 and 282 are the molecular weights of SBMA and BDACT, respectively.)

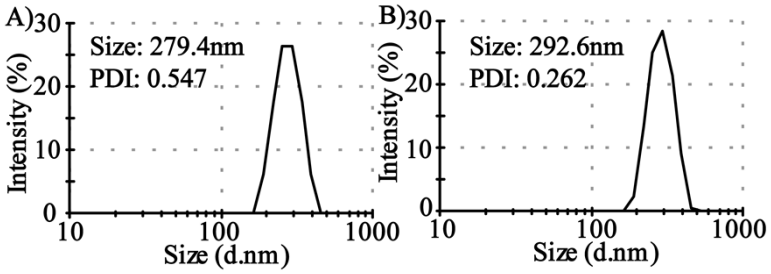


**Figure S2.** Particle size distribution of CS-*g*-PSBMA (A) in PBS and (B) in PBS/fetal bovine serum (v/v = 10/1).

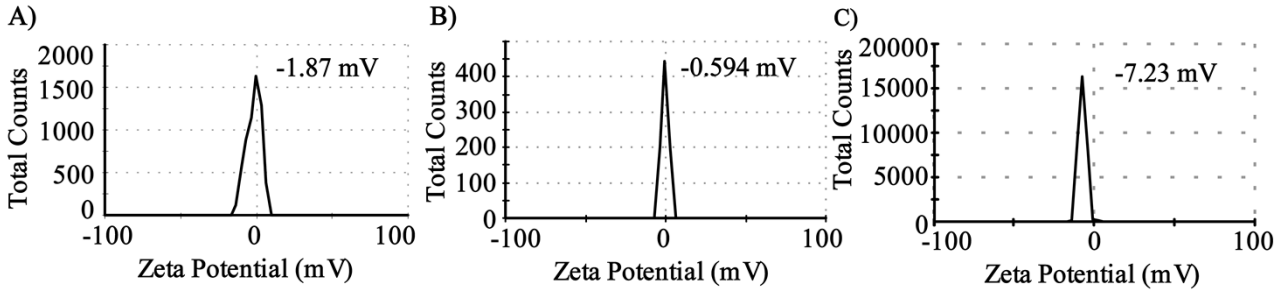


**Figure S3.** The Zeta potentials of (A) CS-*g*-PSBMA nanoparticles, (B) CS(FA)-*g*-PSBMA nanoparticles in PBS, and (C) CS-*g*-PSBMA nanoparticles in PBS/fetal bovine serum (v/v = 9/1). Formula of PBS buffer solution here: NaCl (0.80 g), KCl (0.02 g), Trisodium Citrate (0.10 g), $NaHCO_3$ (0.10 g), and $NaH_2PO_4.2H_2O$ (0.0057 g) were dissolved in water (1 L).

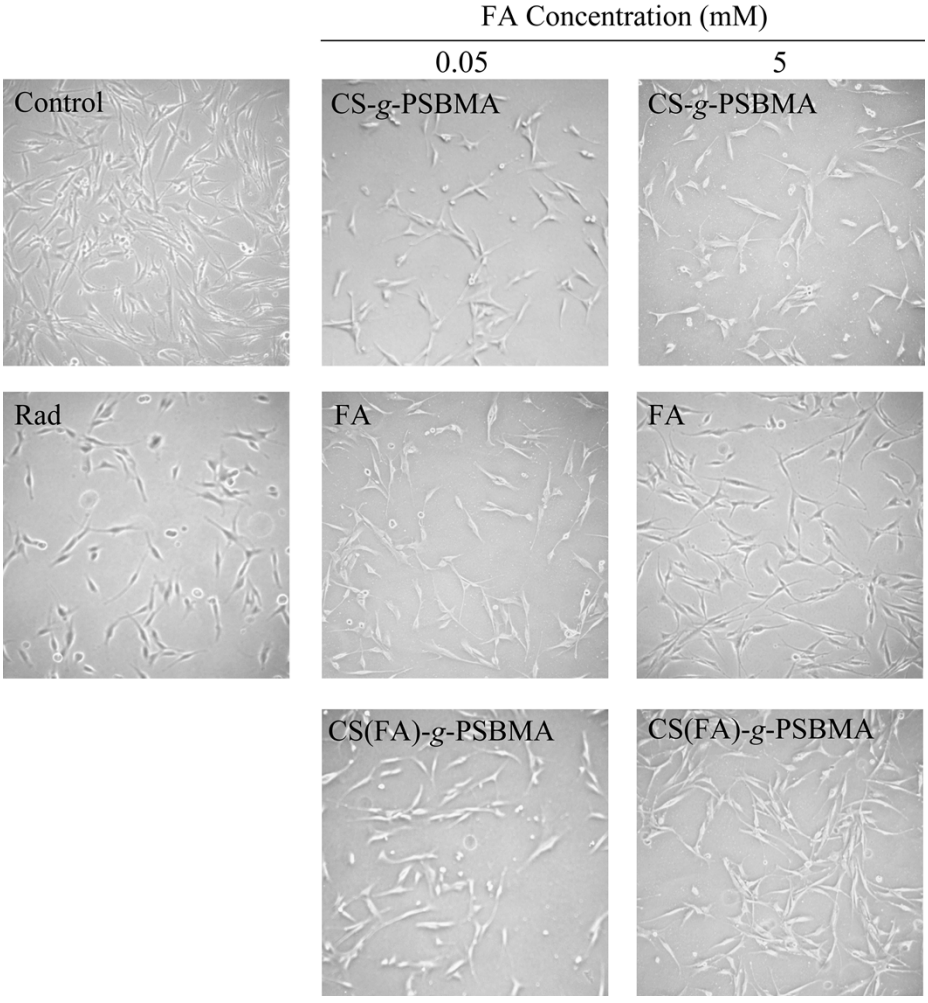


**Figure S4.** The microscopic images for the dependence of relative cell vitality of HDF cells by colorimetric MTT assay on FA concentration for FA, CS-*g*-PSBMA and CS(FA)-*g*-PSBMA nanoparticles, respectively. The concentration of CS-*g*-PSBMA was same as that of CS(FA)-*g*-PSBMA in each group. HDF cells were treated with the drugs for 12 h before radiation, and then were upon γ-radiation exposure (4 Gy).

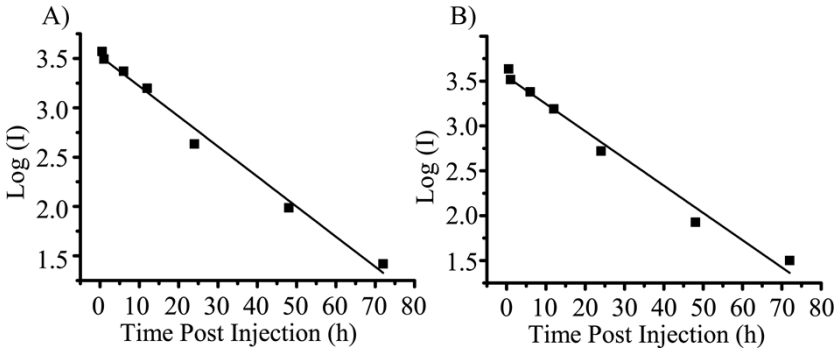


**Figure S5.** The logarithm of fluorescence intensity as a function of time for (A) CS-*g*-PSBMA, and (B) CS(FA)-*g*-PSBMA nanoparticles. $t_{1/2}$ was calculated from the slope (b) of the line according to the formula: $t_{1/2}$ = ln2 / k, and k = -2.303 * b.

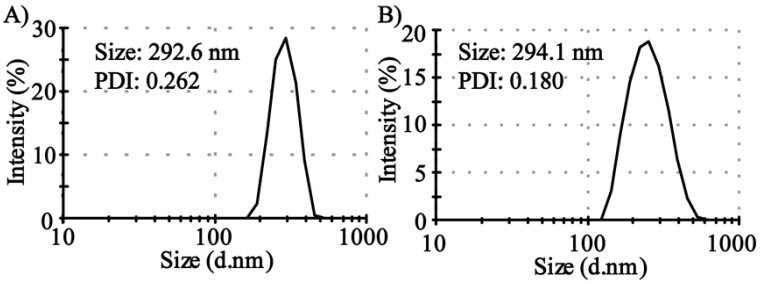


**Figure S6.** Particle size distribution of CS(FA)-*g*-PSBMA in PBS (A) before γ-ray irradiation (4 Gy) and (B) after γ-ray irradiation (4 Gy).

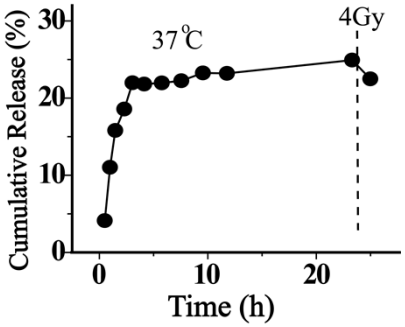


**Figure S7.** In vitro drug release profiles from CS-*g*-PSBMA nanoparticles (loading efficiency = 26.2 %) at 37 °C. At the time of 24 hours, the in vitro drug release was placed with a $^{60}$Co source at the dose rate of 2 Gy/min for 2 min.

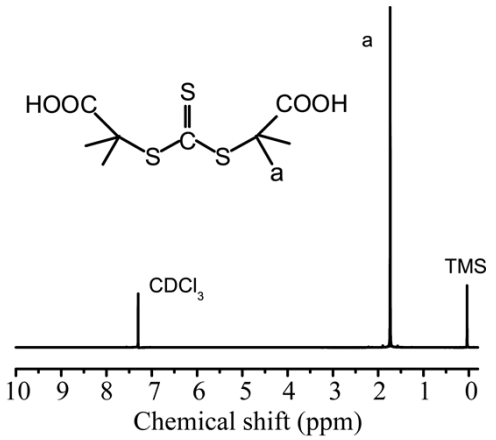


**Figure S8.** $^{1}H$ NMR spectrum (400M, $CDCl_3$) of BDACT.

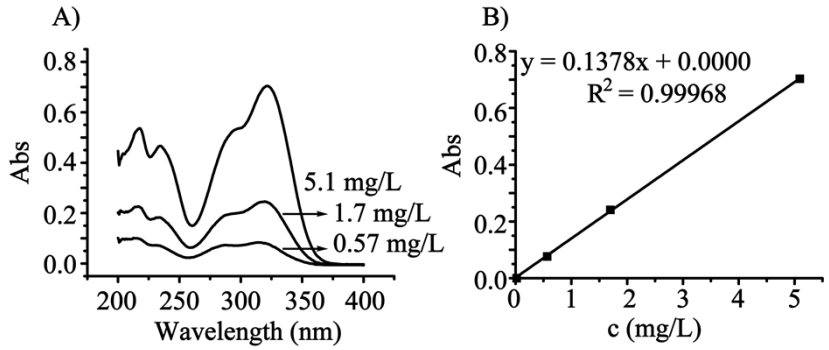


**Figure S9.** (A) The absorbance and (B) the standard curve of FA in ethanol ($\lambda_{max}$= 322.5 nm).

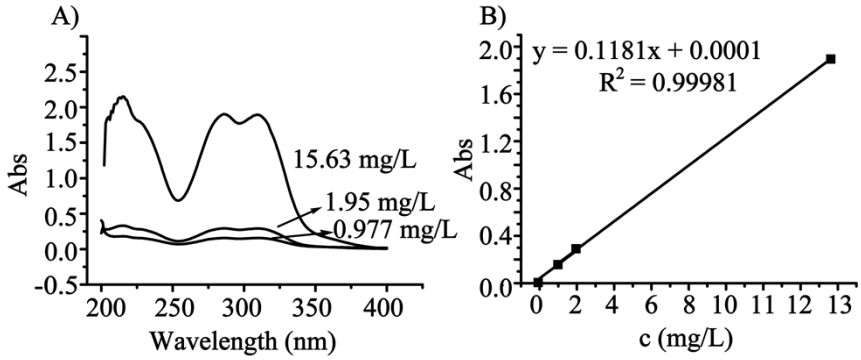


**Figure S10.** (A) The absorbance and (B) the standard curve of FA in PBS ($\lambda_{max}$= 309 nm).

## References

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