

Supplementary Material

Self-assembly of plasma protein through disulfide bond breaking and its use as a nanocarrier for lipophilic drugs†

Shudong Wang, Guangming Gong*, Hua Su, Wenya Liu, Zhen Wang and Li Li

Department of Pharmaceutics, Jinling Hospital, Nanjing University School of Medicine, Nanjing 210002, People's Republic of China.

Tel.: +86-25-80860161; E-mail: wangshdong163@sina.com; gonggm@126.com;

Quantification of sulfhydryl (SH) groups in DTT-reduced albumin

Under different concentrations, albumin (Sigma, USA) was incubated with dithiothreitol (DTT) at 25 and 37 °C. After 2, 5, 15, and 30 min, 4 mL of water suspensions were dialyzed (membrane cutoff MW: 8 K Dalton) for 24 h to remove the remaining DTT. The number of free SH groups was quantified using the Ellman method. Up to 1 mL of albumin solution was mixed with 5 mL of Ellman's reagent (5,5-dithio-bis-(2-nitrobenzoic acid) [DTNB]. After 15 min, the specimens were analyzed at 412 nm using a UV-vis spectrophotometer 1700 (SHIMADZU, Japan). The number of SH in albumin was calculated according to the cysteine standard solution (0, 1, 2, 5, 10, and 20 mM).

For the Bradford method, the specimens were analyzed spectrophotometrically at 595 nm using a UV-vis spectrophotometer. The protein content was calculated relative to the albumin standard solution (Nanjing Jiancheng Bioengineering Institute).

Size and shape of albumin nanoparticles

Albumin was dissolved in 5 mM Tris at 5 mg mL⁻¹ concentration with DTT (Aoduofuni Bio Co., Ltd., China) to a final concentration of 5 mM at 37 °C. After 3, 5, and 10 min, drops from three solutions were placed on a 100-mesh copper grid coated with carbon (Zhongjingkeyi Corporation, China). All specimens were observed using a JEOL JEM-2100 high-resolution electron microscope (HRTEM, Japan) after the grid was air dried.

Fluorescence spectrum and surface hydrophobicity measurement

HSA (0.18-0.10 mg mL⁻¹) was incubated with 20 mM DTT at 37 °C. After 10 min, CCM solution (10% of HSA, w/w) was added, and the fluorescence of the HSA-NPs-CCM and the same concentration of HSA was determined using a fluorescence spectrophotometer F-7000 (HITACHI, Japan). The excitation wavelength was 295 nm, whereas the emission spectrum ranged from 300 nm to 450 nm. To quantify hydrophobicity, 1-(anilino) naphthalene-8-sulfonic acid (Aoduofuni Bio-Corporation, China) was added to the respective albumin solutions, and the mixture was equilibrated for 12 h. The fluorescent spectrum was obtained using a fluorescence spectrophotometer F-7000 (HITACHI, Japan). The excitation wavelength was 390 nm, and the emission spectrum was collected from 400 nm to 650 nm. The measurements were performed in triplicate.

Effect of pH on formation of albumin particles

Up to 4 mg mL⁻¹ of albumin was used for nanoparticle preparation under different pH ranging from 3 to 9 with 20 mM DTT at 37 °C. The nanoparticle sizes were measured with a 90 plus particle size analyzer (Brookhaven Instruments Corporation, USA)

Preparation of albumin-curcumin nanoparticles (NPs-CCM)

Up to 100 mg HSA (20 mL) was incubated with 5 mM DTT at 37 °C for 20 min, and 10 mg of CCM solution (with ethanol as solvent) was added. The NPs were dialyzed for 24 h, and then dropped into the copper grid. HRTEM was used to observe the morphology and size of the NPs.

Encapsulation rate and drug loading rate of NPs-CCM

100 mg (20 mL) HSA was incubated with 5 mM DTT at 25 °C for 20 min, and 5 mg of CCM solution (with ethanol as solvent) was added. The free CCM was isolated from total NPs-CCM by ultrafiltration. The amount of free CCM and that of encapsulated CCM in the NPs was determined by HPLC assay.

The encapsulation rate (%) = Encapsulated CCM in NPs-CCM/Total CCM in NPs-CCM.

The drug loading rate of NPs-CCM was determined with the equation given below:

Drug loading rate (%) = weight of CCM in NPs-CCM/ weight of CCM in NPs-CCM + weight of HSA in NPs-CCM

Circular Dichroism (CD) Spectra.

CD measurements of HSA and HSA-CCM were performed on a JASCO 810 (Japan) using a 1.0 cm quartz cell. The wavelength was from 200 to 260 nm, and the scanning speed was 50 nm min⁻¹. The concentration of HSA, and HSA-CCM solution was 10 mg mL⁻¹ (quantified by Coomassie Kit).

In vitro release of NPs-CCM

The release profiles of NPs-CCM were measured by the dialysis method. Amount of lyophilized NPs-CCM were redispersed in 2 mL of phosphate buffer solution (PBS, pH 7.4) and closed into a dialysis bag (MW cut-off

8000). The bags were dialyzed against 10 mL PBS (containing 5% dimethyl sulfoxide, v/v). At certain time points, the CCM released buffer was collected for HPLC assay, and same volume of releasing buffer was added.

Assembly of DTT-denatured albumin after its free SH is blocked

The DTT-denatured albumin solution was dialyzed for 24 h to remove remaining DTT, and to prepare a 2 mg mL⁻¹ solution. Excessive DTNB was added into this solution to close SH groups. The prepared solution was heated to 37 °C, and 10% paclitaxel (Xianxuanhao Corporation, China, w/w) was added. The size of the albumin-CCM particles was about 180 nm from DLS.

Cellular uptake of NPs-CCM

The NPs-CCM nanoparticles were labeled with fluorescein isothiocyanate (FITC): 20 mg of HSA NPs was dissolved in 3 mL of bicarbonate buffer (0.01 M), and 3 mg of FITC was added. The mixed solution was incubated at 37 °C for 18 h and continuously stirred. The solution was dialyzed with phosphate buffer solution (pH 7.2) for 24 h. FITC-HSA, which was set as control, was prepared as that of NPs-CCM. The preparation of FITC-HSA was the same as that of NPs-CCM.

MCF-7 cells (Chinese Academy of Science, Shanghai, China) were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% FBS, 1% penicillin-streptomycin and incubated in a CO₂ incubator of 5% carbon dioxide at 37 °C. MCF-7 cells were seeded into a confocal dish (Biotech, USA) of 2 × 10⁵ cells/well, and cultured for 24 h. The cells were then incubated with FITC-NPs-CCM suspension at 0.5 mg mL⁻¹ concentration, 37 °C for 2 h, and the suspension was then removed. The wells were washed three times with 200 μL of cold PBS to get rid of traces of nanoparticles. 400 μL of 4, 6-diamidino-2-phenylindole (DAPI, Cell Apoptosis DAPI Detection Kit, KeyGEN, China) was introduced into the sample wells to stain the cells for 10 min at 37 °C and the cells were fixed by ethanol. The sample well was observed by CLSM (ZEISS LSM 710, Germany) with excitation wavelengths of 488 nm for FITC, and 543 nm for DAPI.

Targeting property of NPs-CCM *in vivo*

NPs-CCM (30 mg) was dissolved in 3 mL of Tris buffer (5 mM, pH 7.8), and then 3 mg of NIR-797 (300 μL of DMSO as solvent) was added to the mixture. The resulting solution was incubated at 37 °C for 10 h and continuously stirred. The solution was dialyzed with 0.05 M Tris buffer for 12 h. NIR-797-HSA was prepared in the same way.

NIR-797-HSA solution, NIR-797-NPs-CCM (0.1 mg equivalent NIR-797) were injected into H22 tumor bearing mouse (ICR, 30~32 g, n=4) through the tail vein followed by NIR fluorescence imaging using an IVIS Spectrum (Caliper, USA). The emission wavelength was 800~900 nm and the exposure time was 2 s. Scans were carried out at 1, 28, and 48 h post intravenous administration. After the live imaging, tumor, heart, liver, spleen, kidneys and lung were harvested for images to evaluate the biodistribution of NIR-797 and NIR-797 labelled NPs-CCM nanoparticles. All experiments were performed in compliance with the Management Guidelines for Care and Use of Animals, and these experiments had been approved by the Institutional Animal Experimentation Ethics Committee of the Nanjing General Hospital of Nanjing Military Command.

Immunogenicity of rat serum albumin (RSA)-NPs-CCM

RSA-NPs-CCM were fabricated as that of HSA-NPs-CCM.

Male Sprague-Dawley (SD) rats, weighing 200–220g, were used for the immunization studies. All rats were divided into three groups (n=6) and intravenously treated with RSA (Nanjing Hanyun Company) and RSA-CCM nanoparticles, equivalent to a dose of 60 mg/kg albumin at days 1 to 6. Saline was used as control.

Blood samples were collected from the retro-orbital sinus of rats at days 0 and 30 after the three solutions administered *in vein*. The blood samples were allowed to clot overnight and then centrifuged at 10000 × g for 5 min to collect serum, which was stored at – 20 °C until tested.

Anti-RSA antibodies in serum samples were determined by using enzyme linked immune-sorbent assay (ELISA). Briefly, each well of microtiter plates (Corning, USA) was coated with 100 μL RSA (10 μg mL⁻¹) in carbonate buffer (pH 9.6) and incubated overnight at 4 °C. The plates were washed three times with PBS-Tween 20 (0.05%, v/v) (PBS-T) and blocked with gelatin (2%, w/v) for 2 h at 37 °C, followed by washing with PBS-T. The serum samples were diluted with PBS at 1:1000 and 1:2000. One hundred microliters of the diluted serum samples were added to the wells of coated ELISA plates, and sheep anti-RSA antibody (Santa Cruz Biotechnology, USA) was set as positive control. The plates were incubated for 1h at room temperature and washed three times with PBS-T. One hundred microliters of horse reddish peroxidase (HRP) labeled goat anti-rat IgG (1:10000 dilution, Kangwei Company, China) antibodies were added to well for the determination of IgG titer, respectively. The plates were kept for 1h at room temperature and then washing was repeated. One hundred microliters of tetramethyl benzidine (TMB) solution was added to each well, subsequently color was developed for 15 min. Absorbance of each well was taken at 630 nm using a plate reader (SAFIRE Tecan, USA).

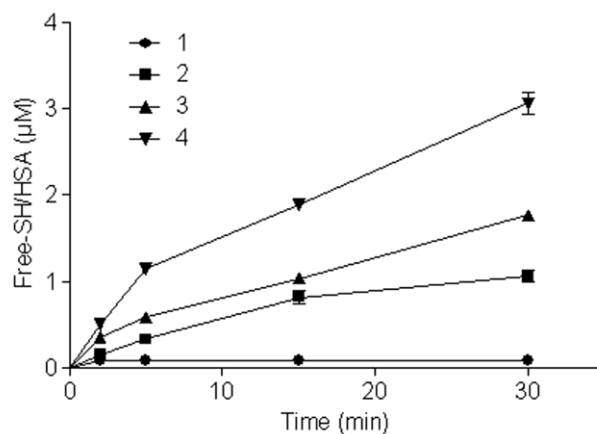


Figure S1. Concentration of free SH groups in HSA with DTT under the different conditions described in Table S1.

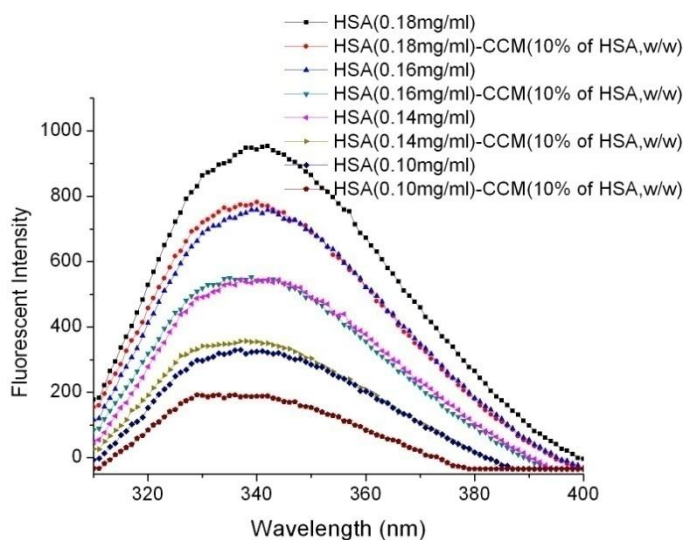


Figure S2. Fluorescent emission spectra of HSA (0.1-0.18 mg mL⁻¹) and HSA-NPs-CCM (10% of HSA, w/w).

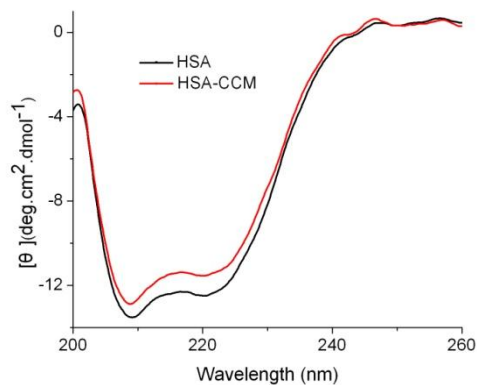


Figure S3. CD spectra of HSA and HSA-NPs-CCM. The spectrum of CCM was subtracted from that of NPs-CCM, and the content of albumin was the same in these two groups.

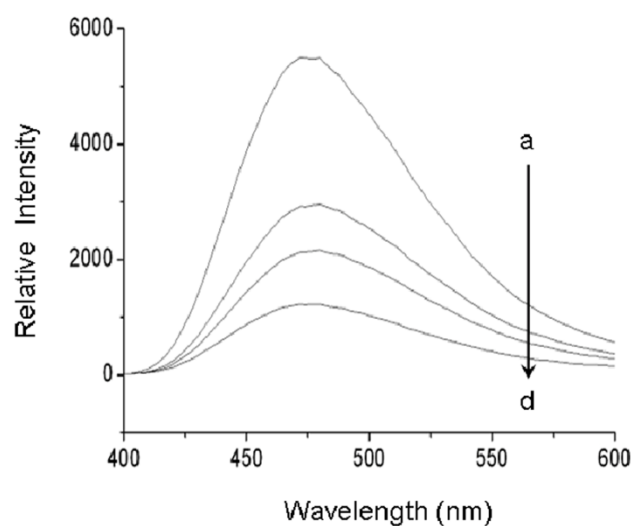


Figure S4. Spectral change of ANS incubated with HSA (2 mg mL^{-1}) reduced under different conditions. a) HSA solution without DTT; b–d) HSA incubated with DTT for 2, 5, and 10 min.

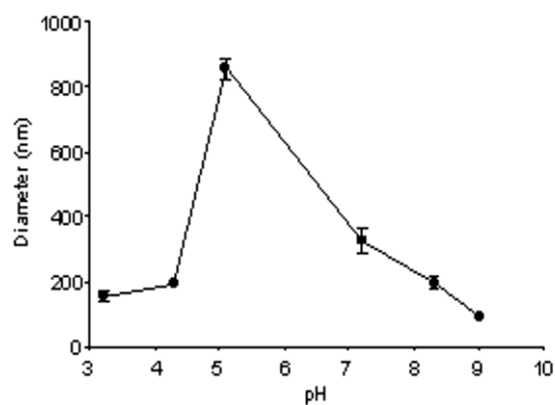


Figure S5. Effect of pH on diameter of HSA NPs incubated with DTT (5 mM) at 37°C .

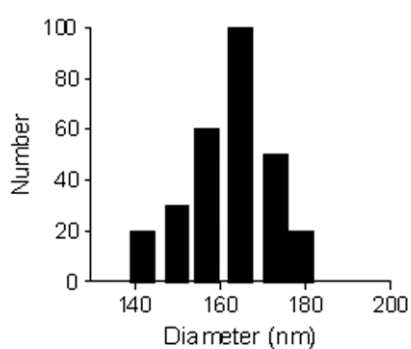


Figure S6. DLS of NPs-CCM fabricated using excessive DTNB as blocking reagent to close the active SH group of HSA molecules reduced with DTT and additional CCM.

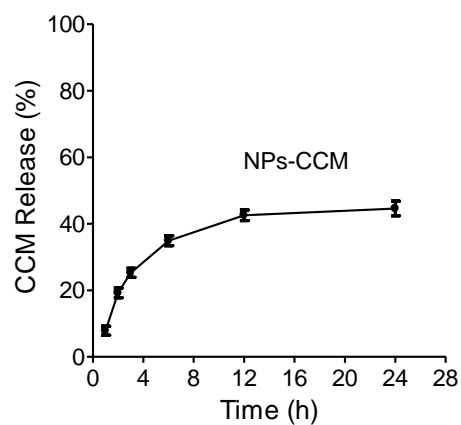


Figure S7. Accumulative CCM release curve from NPs-CCM groups dialyzed against pH 7.4 phosphate buffer at 37 °C. Each data point is represented as mean \pm SD (n=3).

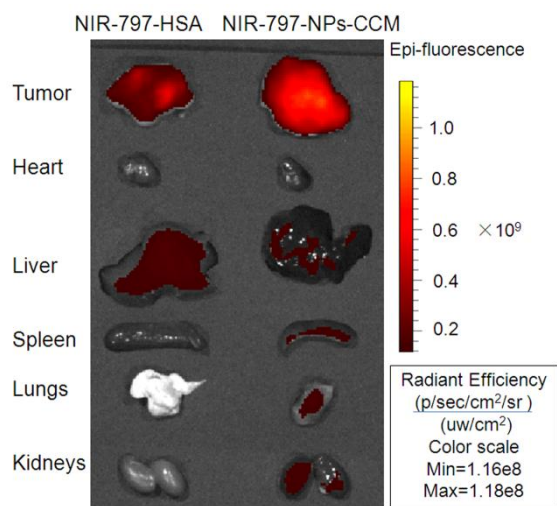


Figure S8. *Ex vivo* images of different tissues of NIR-797-HSA and NIR-797-NPs-CCM administered to H22 tumor-bearing mice after 48 h. The excitation wavelength was 750 nm and the emission wavelength was 850 nm.

Table S1. Conditions used to prepare free SH under different temperatures and concentrations of DTT and HSA.

Number	Temperature (°C)	DTT (mM)	HSA (mg mL ⁻¹)
1	25	0	2
2	25	5	2
3	37	5	2
4	37	5	5

Table S2. The absorbance of plate wells at OD₆₃₀ by ELISA to test the immunogenicity of RSA-NPs-CCM and RSA.

	1#		2#		3#		4#		5#		6#	
	1:1000	1:2000	1:1000	1:2000	1:1000	1:2000	1:1000	1:2000	1:1000	1:2000	1:1000	1:2000
NaCl	0.096	0.111	0.115	0.110	0.114	0.127	0.094	0.111	0.129	0.097	0.104	0.089
	0.106	0.109	0.103	0.101	0.097	0.104	0.096	0.095	0.107	0.090	0.096	0.102
	0.093	0.089	0.105	0.094	0.090	0.094	0.088	0.093	0.102	0.097	0.096	0.093
RSA (negative control)	0.108	0.094	0.116	0.095	0.131	0.119	0.102	0.116	0.114	0.087	0.088	0.105
	0.090	0.096	0.099	0.091	0.117	0.111	0.094	0.107	0.101	0.091	0.084	0.090
	0.089	0.092	0.098	0.096	0.107	0.103	0.092	0.097	0.092	0.082	0.081	0.092
RSA-CCM NPs	0.111	0.114	0.125	0.100	0.092	0.097	0.107	0.102	0.098	0.105	0.089	0.086
	0.097	0.099	0.102	0.093	0.097	0.117	0.098	0.097	0.100	0.103	0.095	0.110
	0.104	0.091	0.113	0.091	0.101	0.100	0.096	0.092	0.106	0.094	0.096	0.087
Sheep anti-RSA	1.620	1.323										
antibody	1.509	1.258										
(positive control)	1.586	1.372										