Electronic Supplementary Material (ESI) for Chemical Communications

Human serum albumin (HSA) nanoparticles stabilized with intermolecular disulfide bonds

Wentan Wang, Yanbin Huang*, Shufang Zhao, Ting Shao and Yi Cheng*

Department of Chemical Engineering, Tsinghua University, Beijing 100084, P. R. China

*Emails: yanbin@tsinghua.edu.cn (Yanbin Huang), yicheng@tsinghua.edu.cn (Yi Cheng)

Supporting Information

Experimental methods

Albumin reduction and determination of the number of the free sulfhydryl

HSA (Sigma, USA, catalog number A1653) was dissolved in deionized water at concentration 20 mg·mL^{-1} with the excessive glutathione (GSH, Kokusan Seiko Inc., Japan, catalog number Rj112) at 37 ºC to break up the intramolecular disulfide bonds and expose free sulfhydryl groups. After 10, 20, 40, 60 and 120 min, 2 ml of solution were taken out to dialyze (membrane cutoff MW: 12K-14K Dalton) in deionized water at 4 ºC for 24 h to get rid of the excessive GSH and probably its oxidized form GSSG. After the dialysis, the amount of the sulfhydryl in the mixture was determined using the Ellman method[1-3], and the protein content of the mixture was determined using the Bradford method[4-5]. For the Ellman method, 250 μL of the mixture and 50 μL Ellman's reagent (5,5'-Dithio-bis-(2-nitrobenzoic acid), J&K Scientific Ltd., China, catalog number LD60L34) solutions were added into 2.5 mL of reaction buffer. After incubating the mixture at room temperature for 15 min, the samples were analyzed spectrophotometrically at 412 nm by using UV/VIS Spectrophotometer (TU-1901, Beijing Puxi Universal Apparatus Co. LLC.

China). The sulfhydryl content of the samples was calculated relative to reference samples, which contained different volumes of a cysteine standard solution and were treated as described before. For the Bradford method, 1 mL of mixture was added into 5 mL of Bradford working reagent (Coomassie brilliant blue G-250, Amresco Inc., USA, catalog number 0615). After incubating the mixture at room temperature for 20 min, the samples were analyzed spectrophotometrically at 595 nm by using UV/VIS Spectrophotometer (TU-1901, Beijing Puxi Universal Apparatus Co., Ltd, China). The protein content of each sample was calculated relative to reference samples, which contained different volumes of a HSA standard solution and were treated as described before.

Based on these results, 50 mM GSH was chosen as the reduction condition for the subsequent studies.

Determination of fluorescent spectrum and surface hydrophobicity

Similarly, HSA was incubated at 40 $mg \cdot mL^{-1}$ in deionized water with 50 mM GSH at 37 ºC. After 10, 20, 40, 60 and 120 min, 2 ml of solution were taken out to dialyze in the deionized water at 4 ºC for 24 h. For the measurement of self-fluorescence of the protein, each sample was detected with a fluorescence spectrophotometer F-2500 (HITACHI, Japan). The excitation wavelength was 295 nm, while the emission spectrum was recorded from 300 to 450 nm. For the measurement of surface hydrophobicity, 1-(anilinon) aphthalene-8-sulfonic acid (ANS) (Shanghai Tixiai Huacheng Industry Development Co. Ltd., China) was added into each sample and the mixture equilibrated for 1 h. Then, the fluorescent spectra of the mixture were detected using fluorescence spectrophotometer F-2500 (HITACHI, Japan). The fluorescence spectra were investigated with an

excitation wavelength of 390 nm. The emission spectrum was collected in the range of 400-650 nm. All the measurements were performed in triplicate.

Preparation of HSA-NPs

80 mg HSA was firstly dissolved in 2 mL deionized water with 50 mM GSH at 37 ºC to break the intramolecular disulfide bonds. Then, 8 mL of the desolvating agent ethanol was added into the solution to supersaturate the HSA solution and precipitate the nanoparticles. The suspension was kept under stirring at 37 ºC for 10 minutes. After that, the suspension was dialyzed (membrane cutoff MW: 12K-14K Dalton) with deionized water at 4 ºC for 24 h to remove ethanol and GSH.

Determination of particle size and size distribution

Average particle size and size distribution were measured by phase analysis light scattering (PALS) using ZetaPALS, Zeta Potential Analyzer (Brookhaven Instruments Corp., USA). The samples were diluted with deionized water and measured at a temperature of 25 ºC and scattering angle 90º.

Scanning Electron Microscope measurement of the HSA-NPs

After dialysis, drops of HAS-NP suspension samples were transferred onto silicon wafer, dried in air and then analyzed by HR-SEM (JSM-7401, JEOL Ltd., Japan). The size of the products was measured using MIVNT image analysis software (Yongheng Optical Instruments Manufacture Co. Ltd., China).

Determination of the amount of has in supernatant after desolvation

For the determination of the percentage of HSA molecules remained in solution, the nanoparticles were separated from the supernatant by centrifugation at 16,000 × g at 4 ºC for 20min. An aliquot

of the supernatant (0.1 mL) was diluted with 0.9 mL water and the amount of the dissolved HSA in the supernatant was determined using Bradford method[4-5] and shown in Figure S6.

Based on these results, case 6 was chosen as basic condition for the subsequent studies, where the yield of the HSA-NPs can be up to 99%.

Determination of pH-dependent particle size and zeta potential

Titration experiments on the HSA-NPs were performed over a pH range between 3 and 10. At 8 predetermined pH values between 3 and 10, the zeta potential of the nanoparticles was measured by microelectrophoresis and the particle size was determined by using ZetaPALS, Zeta Potential Analyzer (Brookhaven Instruments Corp., USA).

Drug Loading and In Vitro Drug Release from the Nanoparticles

Curcumin is a natural polyphenol compound with many important pharmacological properties, which potentially can be used to treat various diseases such as cancers, inflammations, and neurodegenerative diseases. However, its development into clinical uses is limited by the low aqueous solubility. We used our technique to fabricate the curcumin-HSA-nanoparticles (CCM-HSA-NPs) to demonstrate applications of our HSA-NPs.

80 mg HSA was firstly dissolved in 2 mL deionized water with 50 mM GSH at 37 ºC for 1 h. Then, 8 mL of the ethanol (containing 1.5 $mg \cdot mL^{-1}$ curcumin) was added into the solution to precipitate the CCM-HSA-NPs. The suspension was kept under stirring at 37 ºC for 10 minutes. After that, the suspension was dialyzed (membrane cutoff MW: 12K-14K Dalton) with deionized

water at 4 ºC for 24 h to remove ethanol and GSH. The resulted CCM-HAS-NPs were characterized with TEM (Figure S7).

To determine CCM loading in CCM-HSA-NPs, the CCM-HSA-NPs solution was diluted in 5 mL of ethyl acetate/ethanol (9:1, v/v) and sonicated for 30 min to extract CCM completely. CCM levels were determined by high-performance liquid chromatography (HPLC) using a VP-ODS C18 column (150 mm×4.6 mm, 5μm particle size) at 30 ºC. The mobile phase consisted of 80% methanol, 19% water and 1% ethylic acid. The system was run at a flow rate of 0.7 $mL \cdot min^{-1}$ and CCM was detected at 428 nm. CCM loading was defined as CCM content (%, w/w) = (CCM weight in nanoparticles/total nanoparticle weight)×100. All the measurements were performed in triplicate

To determine CCM release profile, 0.5 mL CCM-HSA-NPs solution was closed into a dialysis bag (membrane cutoff MW: 1K Dalton). Then the bag was immersed into 50 mL releaseing solution (50 vol% ethanol solution). At certain time points, the release solution was withdrawn for UV/VIS Spectrophotometer analysis, and same volume of releasing solution was added. The results were shown in Figure S8.

Table S1 Experimental condition for each case under different temperatures and concentrations.

Case number	Temperature, ºC	Concentration of GSH, mM	Concentration of HSA, mg·mL^{-1}
case 1	37	0	20
case 2	37	20	20
case 3	50	20	20
case 4	25	50	20
case 5	37	50	20
case 6	37	50	40
case 7	37	50	10

Supplementary figures

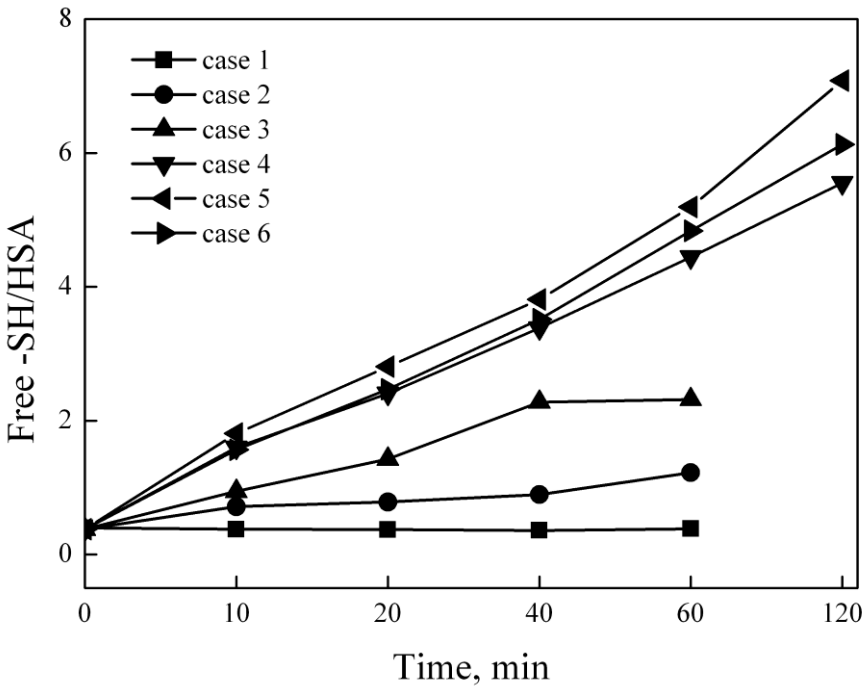

Figure 1 Number of free sulfhydryl groups in HSA under different experimental conditions as specified in Table S1.

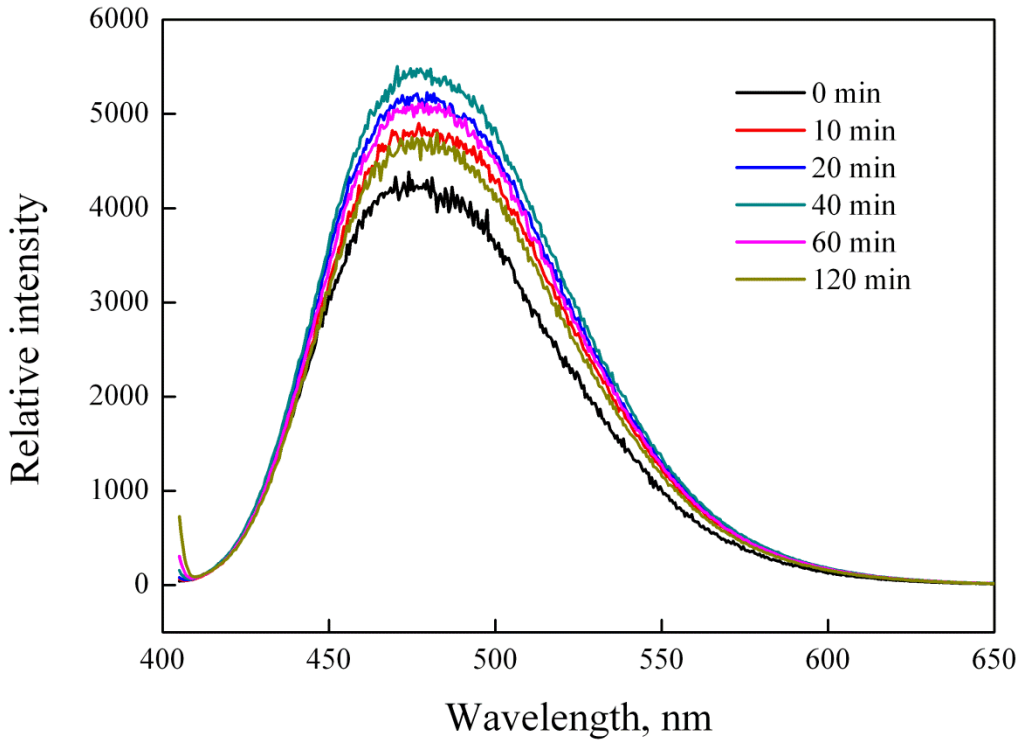

Figure S2 Spectral changes of ANS fluorescence of the reduced HSA under different incubating time. Experimental conditions: 20 mg·mL^{-1} HSA incubated with 50mM GSH at 37 ºC for 10, 20, 40, 60 and 120 min. Each solution was dialyzed with deionized water at 4 ºC for 24 h.

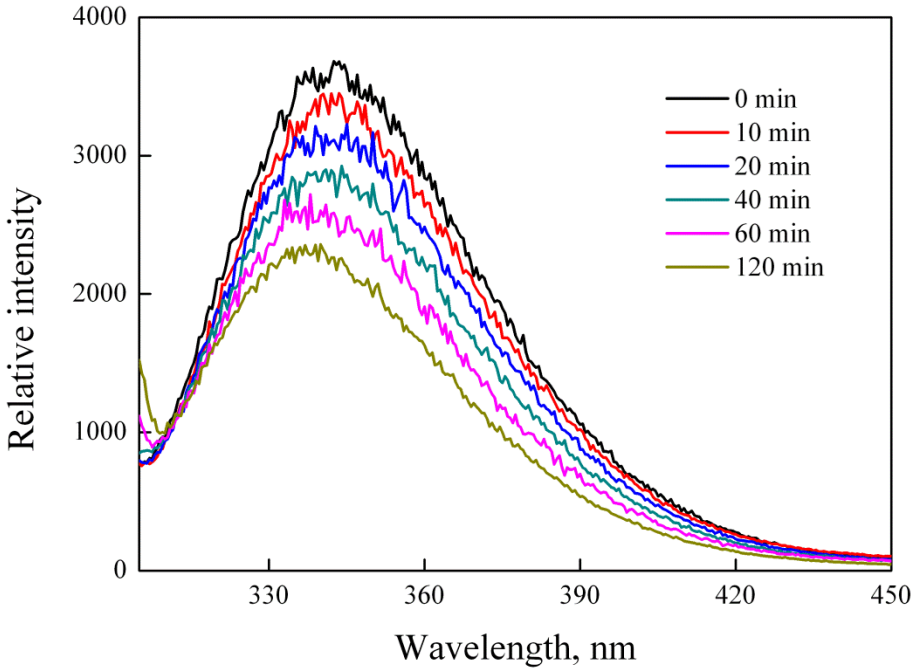

Figure S3 Fluorescence spectra change of reduced HSA under different incubating time. Experimental conditions: 20 mg·mL^{-1} HSA incubated with 50mM GSH at 37 ºC for 10, 20, 40, 60 and 120 min. Each solution was dialyzed with deionized water at 4 ºC for 24 h.

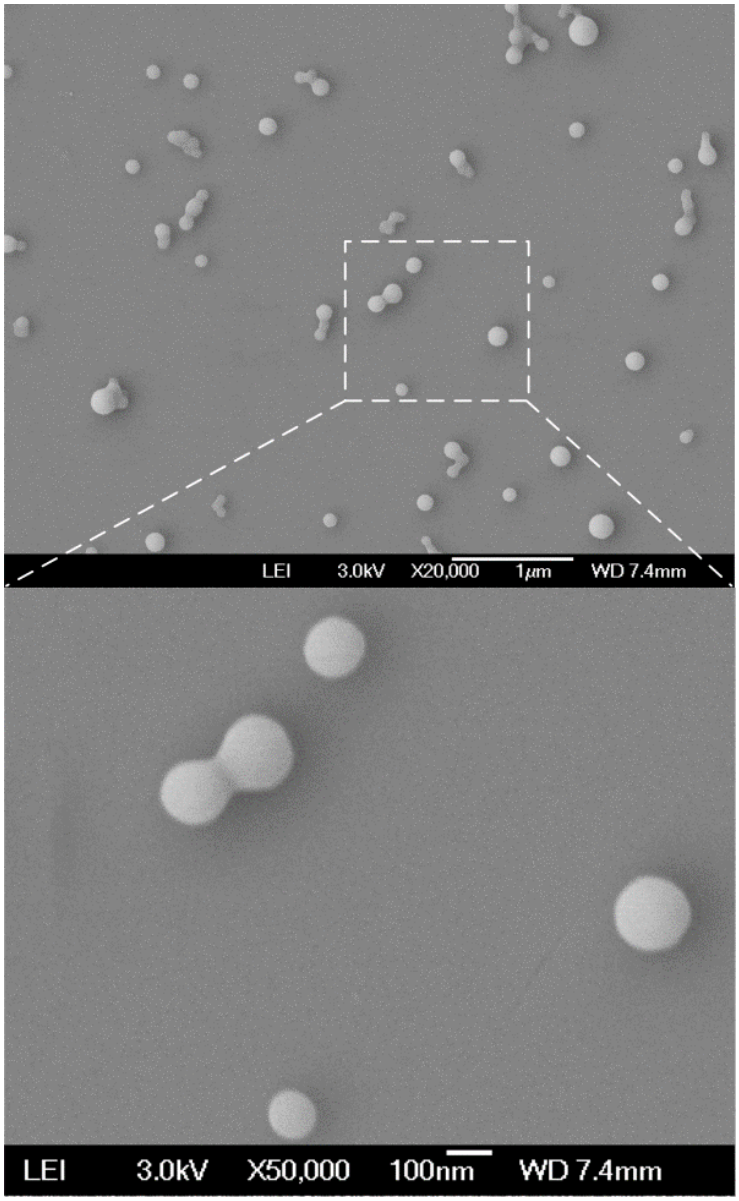

Figure S4 SEM images of the HSA-NPs.

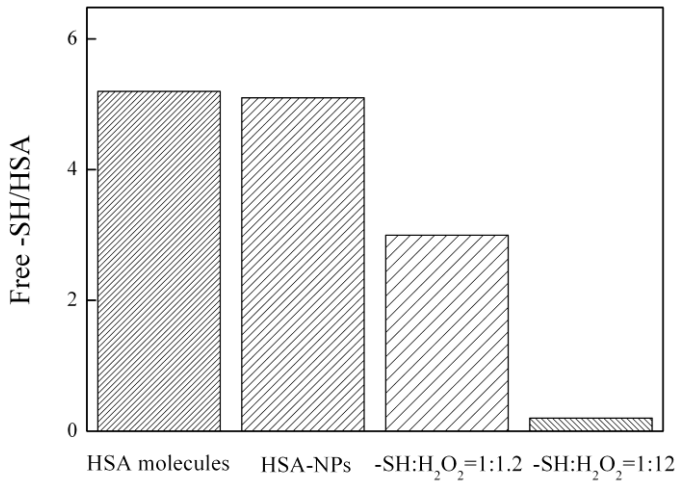

Figure S5 Comparison of the number of the free sulfydryl per HSA molecule. The first column is the HSA molecule before precipitation step; the second is the HSA-NPs after dialysis step; The third and fourth columns are for the H_2O_2-treated HSA-NPs.

There is almost no change in the average number of the free sulfhydryl in each HSA molecule before and after the precipitation step. This phenomenon indicates that the mechanism 2 is the dominant factor in the thiol-disulfide kinetics, which means that the intermolecular disulfide bonds in the HSA nanoparticles are mostly formed by the exchange of the sulfhydryl between the free sulfhydryl and the disulfide bonds. When H_2O_2 used as the oxidant, it can oxidize the sulfhydryl to the disulfides without further oxidization at pH = 8.0 within 20 min[6]. With different amount of the H_2O_2, the remaining sulfhydryl can be oxidized to the disulfide bonds in different extent. When the H_2O_2 is in much excessive, almost all the free sulfhydryl in HSA nanoparticles can be oxidized to the disulfide bonds in less than 10 min under room temperature.

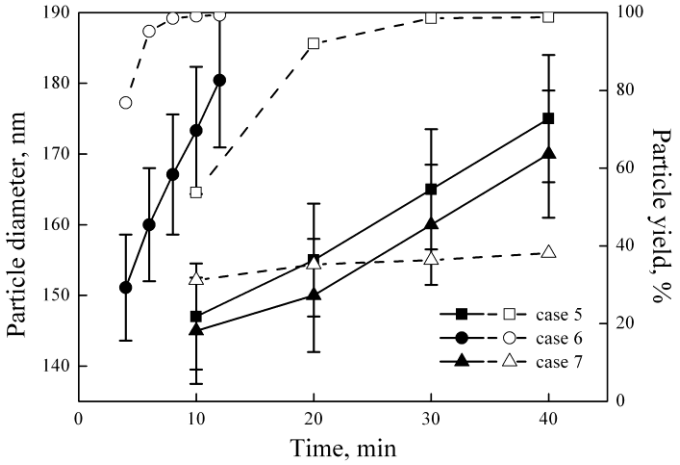

Figure S6 Influence of HSA concentrations on the diameter (solid) and yield (hollow) of HSA -NPs.

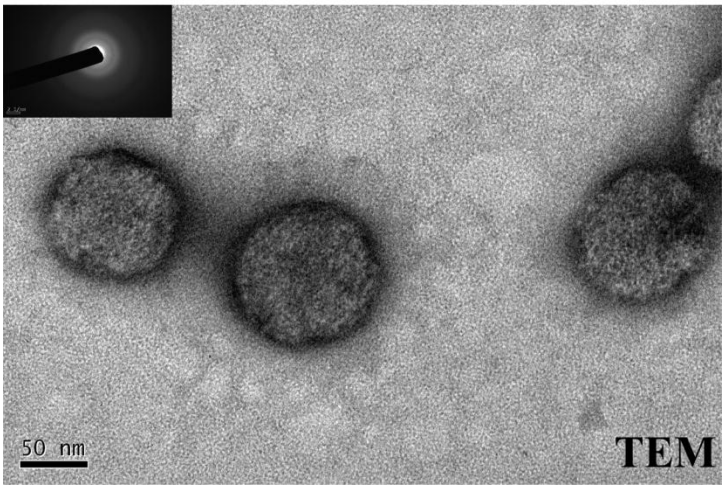

Figure S7 TEM image of the CCM-HSA-NPs.

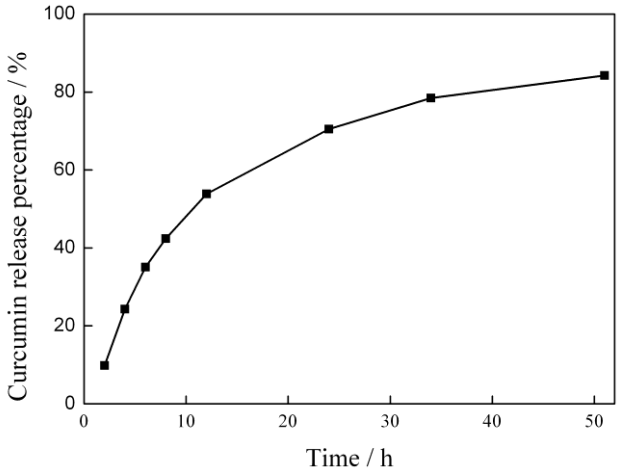

Figure S8 In vitro release curve of CCM from CCM-HSA-NPs at 20 ℃. The CCM loading in CCM-HSA-NPs is 11.3%.

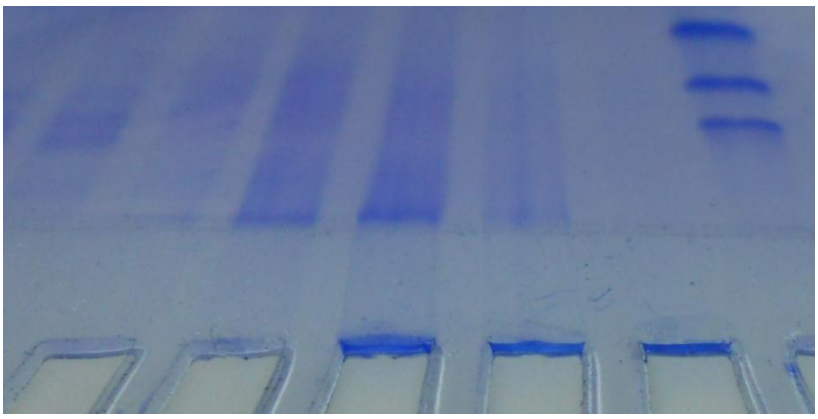

Figure S9. The picture taken from the top of the gel. The lanes from the right to the left are a, b, c, d, and e in Figure 4, respectively.

References

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