# Electronic Supplementary Information

# Mitochondria Targeted Selenium Nanoparticles Enhance Reactive Oxygen Species Mediated Cell Death

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# 1 Experimental Section

### 1.1 Functionalization of HSA

TPP was labeled to HSA for mitochondria-targeting ability according to the previous literature[1]. Briefly, certain amounts of TPP, NHS, and EDC·HCl were mixed in 1.0 mL of DMF, and stirred overnight at room temperature under nitrogen atmosphere. Then 40 mg of HSA dissolved in PBS with a concentration of 1.0 mg/mL was added, followed by continuous stir at room temperature for another 12 h. The molar ratio of TPP : NHS : EDC·HCl was always 1 : 1.4 : 1.12, and molar ratio of HSA : TPP was in a range from 1 : 20 to 1: 100 to obtain HSA labeled with different units of TPP. For example, in a typical synthesis, 40.0 mg of HSA, 12.9 mg of TPP, 4.8 mg of NHS, and 6.4 mg of EDC·HCl were used (molar ratio was 1 : 50 : 70 : 56). After that, the mixture solution was ultrafiltered for three times to remove the unreacted small molecules at around 2800 rpm (815 g) for 30 min (30 kDa), followed by freeze-drying for around 18 h. The prepared protein was stored at 4 °C. Note that, the number of TPPs on HSA can be controlled by reaction ratio between TPP and HSA, but there is slight batch-to-batch variation in each preparation. Therefore, it is necessary to always perform the MALDI-TOF MS (see Section 1.3 and Table S1 in this ESI) and SDS-PAGE (see Section 1.4 and Figure S2 in this ESI) studies to determine the number of TPPs after each synthesis.

FITC was labeled to HSA and HSA-TPP for fluorescent observation on confocal laser scanning microscope. For preparation of HSA-FITC, 46.7 mg of HSA (0.700 μmol) was dissolved in 25 mL of 1× PBS (pH 7.80), with a concentration of about 2.0 mg/mL. Then 2.726 mg of FITC (7.0 μmol) dissolved in 1 mL of 1× PBS (pH 7.80) was added by drops, followed by another 12-hour stirring at room temperature for about 12 h. The molar ratio of HSA : FITC was 1:10. After that, the mixture solution was ultrafiltered for three times with ultrapure water to remove the unreacted small molecules at around 3000 rpm (936 g) for 60 min (30 kDa) each time, followed by lyophilization for around 18 h. The prepared protein HSA -FITC was stored at 4 °C.

For preparation of HSA-TPP-FITC, 46.7 mg of HSA (7.0 μmol) was firstly labeled with TPP as described above by adding certain amounts of TPP, NHS and EDC·HCl dissolved in 1.0 mL of DMF. After stirring for 12 h of TPP labeling, 2.726 mg of FITC (7.0 μmol) dissolved in 1 mL of 1× PBS (pH 7.80) was added by drops without remove of excessive TPP, followed by another 12-hour stirring at room temperature. The molar ratio of HSA : FITC was 1 : 5. After that, the mixture solution was ultrafiltered for three times with ultrapure water to remove the unreacted small molecules at around 3000 rpm (936 g) for 60 min (30 kDa) each time, followed by lyophilization for around 18 h. The prepared protein HSA-TPP-FITC was stored at 4 °C.

**1.2 Zeta potential measurements of proteins**

Protein HSA and HSA-TPP were dissolved in ultrapure water with concentration of 1 mg/mL. Protein solution were filtered through a 0.22 μm ultrafiltration membrane before measured. Zeta potential data are collected in range of –150 – 150 mV, and calculated by Zetasizer Software.

**1.3 MALDI-TOF mass spectra measurements of proteins**

Protein HSA (or HSA-TPP, HSA-TPP-FITC) was dissolved in ultrapure water with concentration of 2 mg/mL, and mixed with sinapic acid matrix with ratio of 1 : 1 (v/v). Solvent of matrix includes 50 % acetonitrile, 50 % proteomics grade water and 0.1 % trifluoroacetic acid (TFA). Mixture solution is dropped on 384-well microplate in 1 μL/well. Molecular weight data are collected in range from 10–300 kDa with a fixed laser intensity of 5500.

**1.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis**

Proteins were dissolved in ultrapure water with 10 μg/mL. 5 μL of protein aqueous solution was mixed with 1 μL of 6× loading buffer, and heated at 90 °C for 5 min before loading. A 7 % denatured SDS-PAGE analysis was carried out in Gly-tris buffer at 90 V constant voltage for about 1.5 h. Gel was imaged under UV laser without any staining, then stained with Coomassie Brilliant Blue R250 (CCB) for 20 min and destained for 6 h, followed by imaging under white light.

**1.5 Detailed synthesis of SeNPs**

In this work, total volume and the ratio of $Na_2SeO_3$ to GSH, HSA, and NaOH should be strictly controlled for preparing SeNPs with suitable size of about 20 nm, good dispersity, and good size distribution. Different ratios of each reagent and different amounts of reaction solution were tried to optimize preparation of SeNPs. At last, it was found the optimal condition is using 3.125 μmol of $Na_2SeO_3$, 0.1875 μmol of protein HSA or functionalized HSA, 25 μmol of reduced GSH, 32.5 μmol of NaOH, and reaction for 30 min at 500 rpm. Here HSA solution was not added in only one time, but added 1/5 of HSA aqueous solution, which was 0.25 mL of HSA, before reaction. Then remaining 1.0 mL of HSA solution was dropwise added during the reaction, followed by another 30-min stirring. Through this way, prepared SeNPs show better dispersity and morphology.

Apart from synthesis procedure, morphology of SeNPs also depends on the water solubility of proteins used as stabilizer. Proteins with better water solubility show more advantages in synthesis, because insoluble proteins will make nanoparticles

aggregate during preparation. Here, TPP groups are labeled on protein HSA for mitochondria targeting. However, TPP units labeling also leads to lower solubility of proteins, which adverses to SeNPs dispersity and size distribution. Thus, proteins HSA labeled with different TPP amounts were tried out to optimize range of TPP units amount, and reach the balance between mitochondria targeting ability and SeNPs morphology. In this work, HSA labeled with TPP amounts in the range from 6 to 23 were applied to synthesize SeNPs.

**1.6 TEM imaging analysis for SeNPs**

Copper grids were pre-treated with plasma for 45 s. SeNPs were diluted with ultra-pure water for a concentration of 400 μg/mL. 10 μL of SeNPs was added on copper grid for 10 min, followed by staining with $UO_2(CH_3COO_2)_2$ (3 %) for 45 s. Copper grid was blot up and dried overnight before imaging in TEM.

**1.7 Cell culture**

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Multicell) with 10% fetal calf serum (FBS) and 1% penicillin streptomycin (10000 IU penicillin and 10000 μg/mL streptomycin, Multicell) in a culture flask at 37 °C in a humidified atmosphere containing 5% $CO_2$. CHO-K1 cells were cultured similarly in Dulbecco's Modified Eagle/Ham's F-12 medium (DME/F-12, HyClone).

**1.8 CellTiter-Glo luminescence cell viability assay**

The cytotoxicity of functionalized HSA or coated SeNPs for HeLa cells or CHO-K1 cells was tested by CellTiter-Glo luminescence assay. HeLa cells ($4.0 \times 10^4$ $mL^{-1}$) were seeded in a 96-well microplate (0.2 mL/well) for 48 h. Then 100 μL of fresh medium with and without SeNPs (different concentration) was replaced to each well. After 24-h incubation, 100 μL of CellTiter-Glo reagent was added to the 100 μL of medium in the wells, followed by a 2-minute vibration and 10-minute incubation at room temperature. The luminescence of this 96-well plate was recorded by a microplate reader with an integration time of 1.0 s per well. The data from a representative of at least three experiments were shown as a percentage of mean ± SD.

**1.9 ROS production detection by flow cytometry assay**

The ROS production of SeNPs in HeLa cells was also detected by flow cytometry assay.

Flow cytometry assay: HeLa cells ($4.0 \times 10^4$ $mL^{-1}$) were seeded in a 24-well microplate (1.0 mL/well) for 48 h. Then 1.25 mL of fresh medium with and without SeNPs (4.0 μg/mL) was replaced to each well. After 24-h incubation, the mixture was

removed. Cells were incubated with 1.0 mL of PBS containing 10 μM of DCFH-DA for 30 min, then washed once using PBS buffer before measurement on flow cytometer (Beckman Coulter, CytoFLEX, excitation wavelength: 488 nm, emission wavelength: 525/40 nm).

**1.10 Cellular uptake efficiency between different cell lines**

SeNPs cellular uptake between different cell lines was tested through fluorescent intensity.

HeLa cells and CHO-K1 cells ($4.0 \times 10^4$ $mL^{-1}$) were seeded in a 96-well microplate (0.2 mL/well) respectively for 48 h. Then 100 μL of fresh medium with HSA-TPP-FITC@SeNPs (10.0 μg/mL) was replaced to each well. After 24-h incubation, the mixture was removed. Cells were washed by PBS for once, followed by fluorescent intensity collection in microplate reader, excitation wavelength: 492 nm, emission wavelength: 525 nm.

## 2 Results

### 2.1 Calculation and characterization of protein HSA-TPP

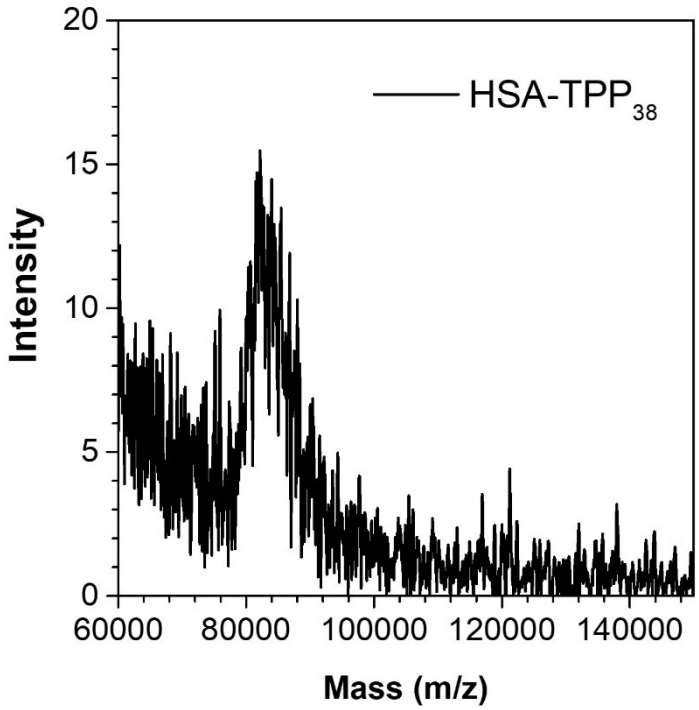


**Figure S1.** MALDI-TOF-MS characterization of HSA-$TPP_{38}$. After reaction with TPP, molecular weight of HSA increases to 82500, indicating about 38 TPP units are labeled to HSA.

**Table S1.** Molecular weights and functionalized group amounts of proteins HSA, HSA-TPP, HSA-FITC, and HSA-TPP-FITC.

| Protein | Molecular Weight / Da | Functionalized Group | |
|---|---|---|---|
| HSA | 66723 | / | / |
| HSA-$TPP_6$ | 69347 | / | 6-TPP |
| HSA-$TPP_{14}$ | 72372 | / | 14-TPP |
| HSA-$TPP_{16}$ | 73112 | / | 16-TPP |
| HSA-$TPP_{23}$ | 76196 | / | 23-TPP |
| HSA-$TPP_{38}$ | 82500 | / | 38-TPP |
| HSA-FITC | 68225 | 4-FITC | / |
| HSA-$TPP_{22}$-FITC | 77277 | 4-FITC | 22-TPP |

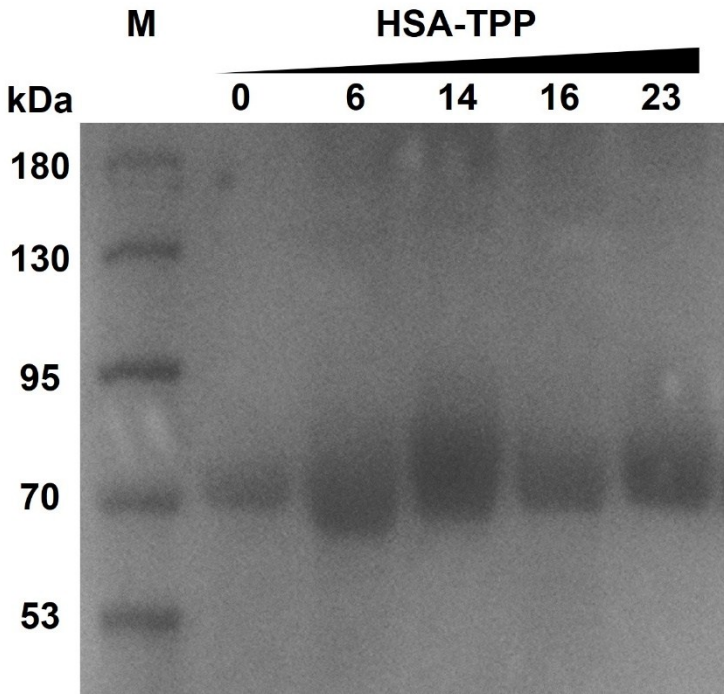


**Figure S2.** SDS-PAGE analysis of proteins native HSA and TPP functionalized HSA. Amounts of TPP units labeled in each kind of protein are marked above lanes. Lane M: commercial protein marker, molecular weights (kDa) are marked beside marker lane.

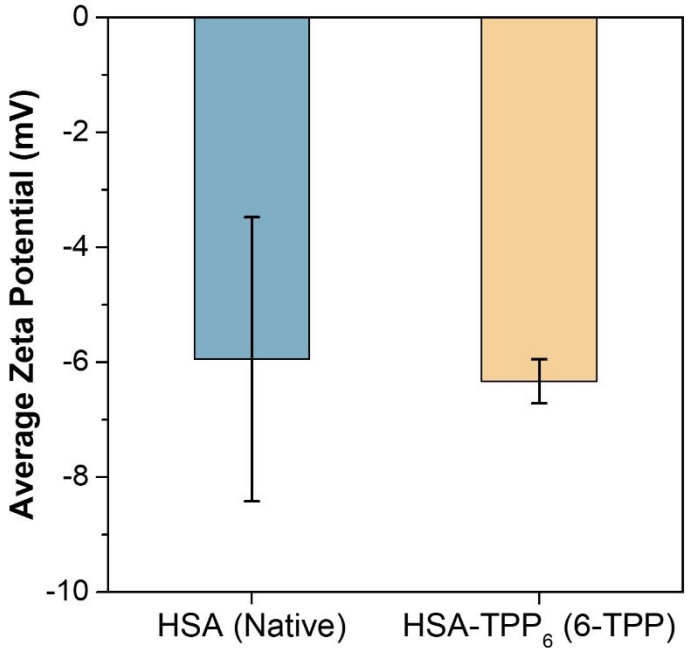


**Figure S3.** Zeta potential characterization of HSA and HSA-$TPP_6$ (labeled with 6 TPP units). Error bar indicates standard deviation (SD) of triplicate tests.

## 2.2 Calculation and characterization of TPP-functionalized SeNPs

Concentrations of SeNPs ($c_{SeNPs}$) are calculated through absorbance at 258 nm. Absorbance of SeNPs dispersed in water at 258 nm is corrected to the absorbance with optical path length of 1 cm ($A_{258}$).

$c_{SeNPs}$ (μg/mL) = $A_{258}$ × 16.689

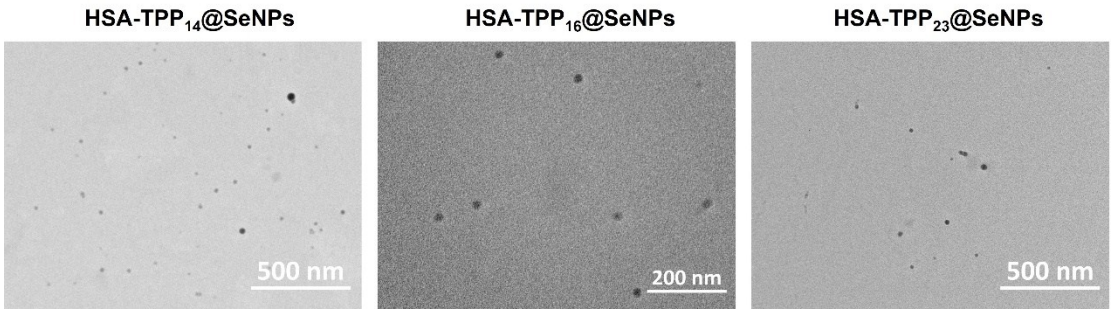


**Figure S4.** TEM images of SeNPs coated with proteins HSA-$TPP_{14}$, HSA-$TPP_{16}$, and HSA-$TPP_{23}$. Scale bar: 200 nm & 500 nm.

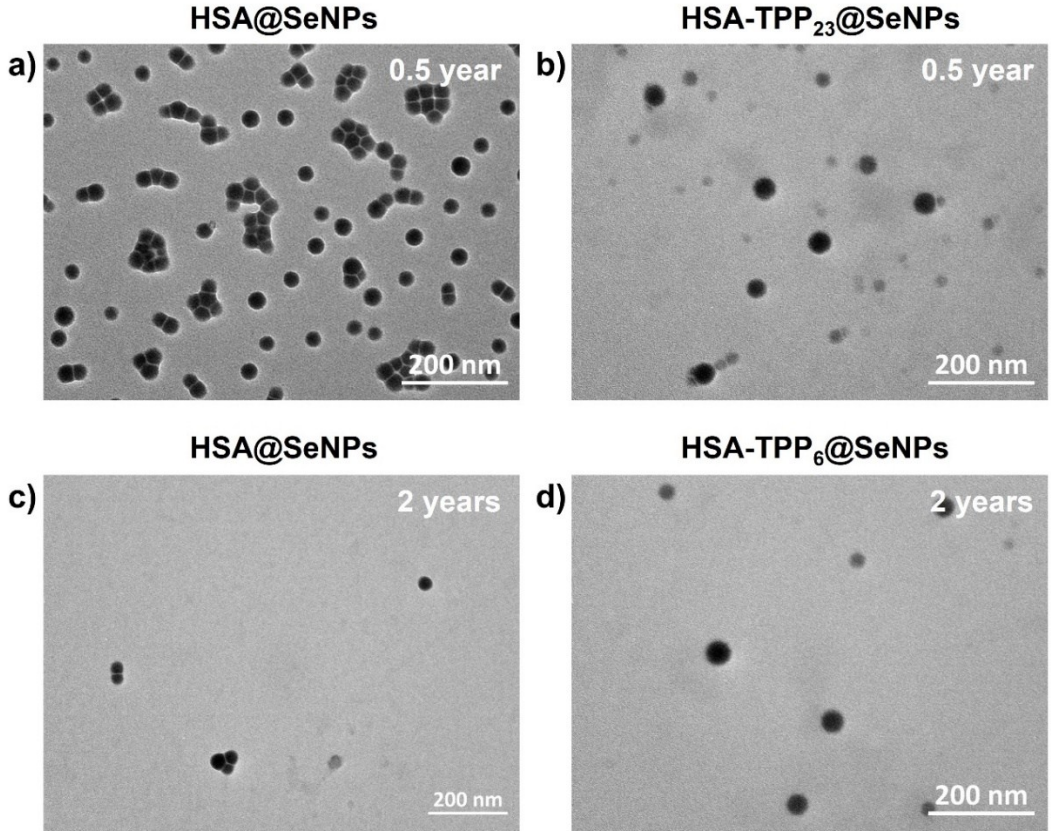


**Figure S5.** TEM images of HSA@SeNPs and HSA-TPP@SeNPs after half to two years. Scale bar: 200 nm.

## 2.3 Comparison of SeNPs uptake in different cell lines

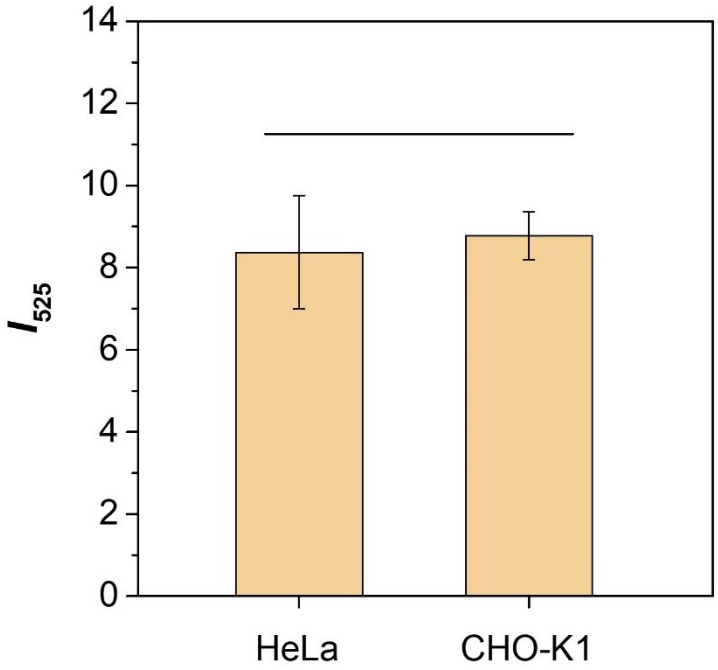


**Figure S6.** Fluorescent intensity at 525 nm of HSA-TPP-FITC@SeNPs in different cell lines. Excitation wavelength: 492 nm. Error bar indicates standard deviation (SD) of triplicate tests. $p > 0.5$, no significant difference.

## 2.4 Characterization of proteins HSA-TPP & HSA-TPP-FITC

Concentration of proteins ($c_{HSA}$) and amounts of FITC units labeled to protein ($R_{FITC/HSA}$) were calculat6ed from absorbance at 280 nm ($A_{280}$) and 492 nm ($A_{492}$).

$c_{HSA}$ (μM) = ($A_{280}$ – 0.1398) * 1000 / (0.5688 * 66.723) (Calculated from standard curve)

$R_{FITC/HSA} = A_{492} * 1000 / (78 * c_{HSA})$

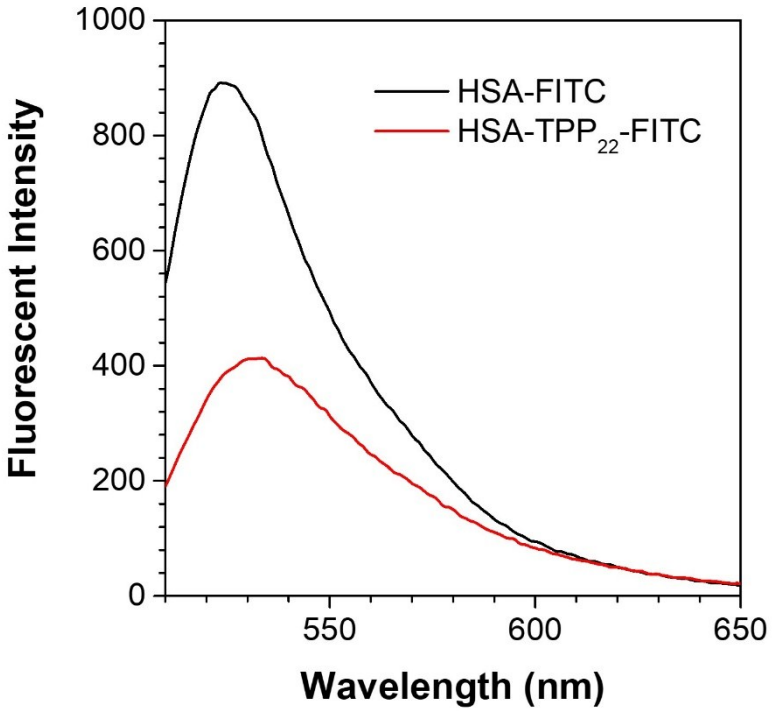


**Figure S7.** Fluorescence emission spectra of proteins HSA-FITC and HSA-$TPP_{22}$-FITC. Excitation wavelength: 492 nm. These two emission spectra show peaks at about 518 nm, proving FITC groups have been labeled to these proteins.

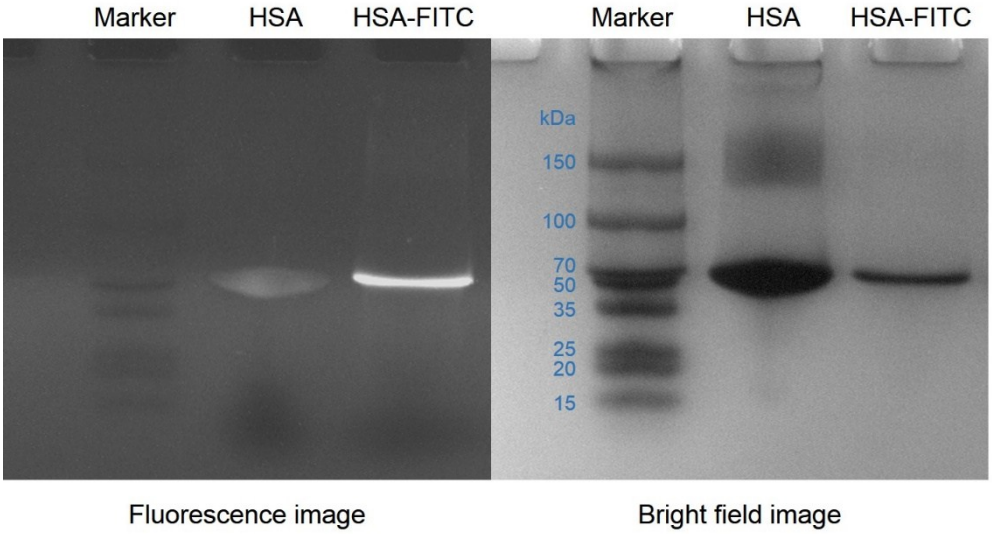


**Figure S8.** SDS-PAGE analysis of proteins HSA and HSA-FITC. After electrophoresis, Gel is imaged under UV light at 302 nm without additional staining. Then this gel is stained by Coomassie Brilliant Blue R250, and imaged again under visible light. Proteins can be detected both in lanes HSA and HSA-FITC, while fluorescence can only be observed in band of HSA-FITC, indicating FITC group is labeled at HSA.

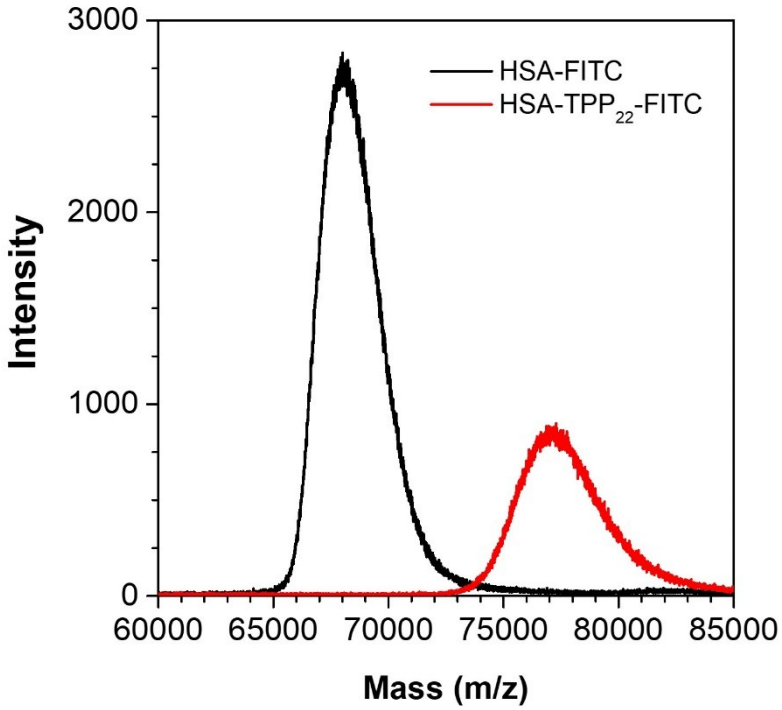


**Figure S9.** MALDI-TOF-MS characterization of HSA-FITC and HSA-$TPP_{22}$-FITC. After reaction with TPP and FITC, molecular weight of HSA increases from 66723 to 77277, indicating about 22 TPP units are labeled to HSA-FITC.

### 2.5 Characterization of TPP and FITC functionalized SeNPs

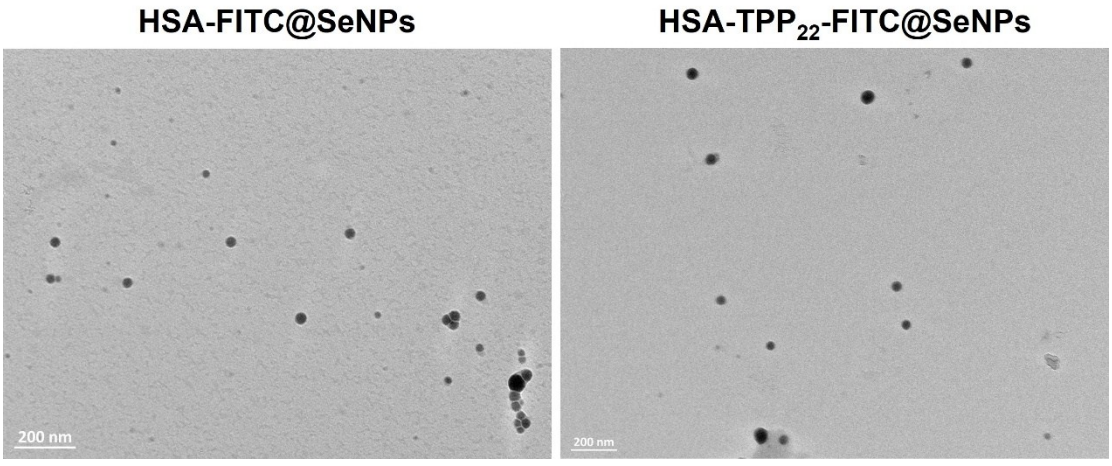


**Figure S10.** TEM images of SeNPs coated with proteins HSA-FITC and HSA-$TPP_{22}$-FITC. Scale bar: 200 nm.

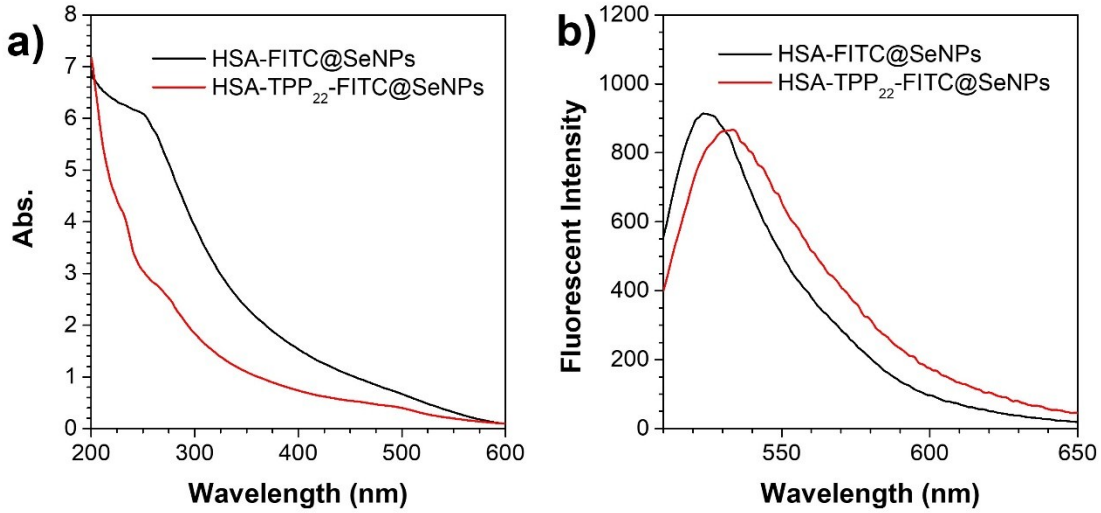


**Figure S11.** Absorption (a) and fluorescence intensity (b, excitation wavelength: 492 nm) spectra of HSA-FITC@SeNPs and HSA-$TPP_{22}$-FITC@SeNPs.

**2.6 Stability of protein-coated SeNPs inside cells**

In the experiment inside cells, fluorescence of SeNPs is indeed from HSA, which is covalently labeled with FITC. To prove SeNPs was still coated with HSA and FITC after a 24-h incubation in cells, HeLa cells were pyrolyzed in RIPA lysis buffer for 30 min, then SeNPs were added into the solution with the final concentration of 4 μg/mL. After 24-h incubation, SeNPs were purified through centrifugation, and resuspended in RIPA lysis buffer without cells. Fluorescence of FITC before and after centrifugation was then compared as shown below. After 24-h incubation and purification, fluorescence of SeNPs without and with TPP only decrease by 16.3% and 27.7%, similar to decrease percentages of SeNPs in lysis buffer (no cells), which are 43.2% and 22.4%. The results indicate after incubation, HSA still coats on SeNPs, and can be separate along with SeNPs under centrifugation. Thus, HSA@SeNPs are stable enough in living cells.

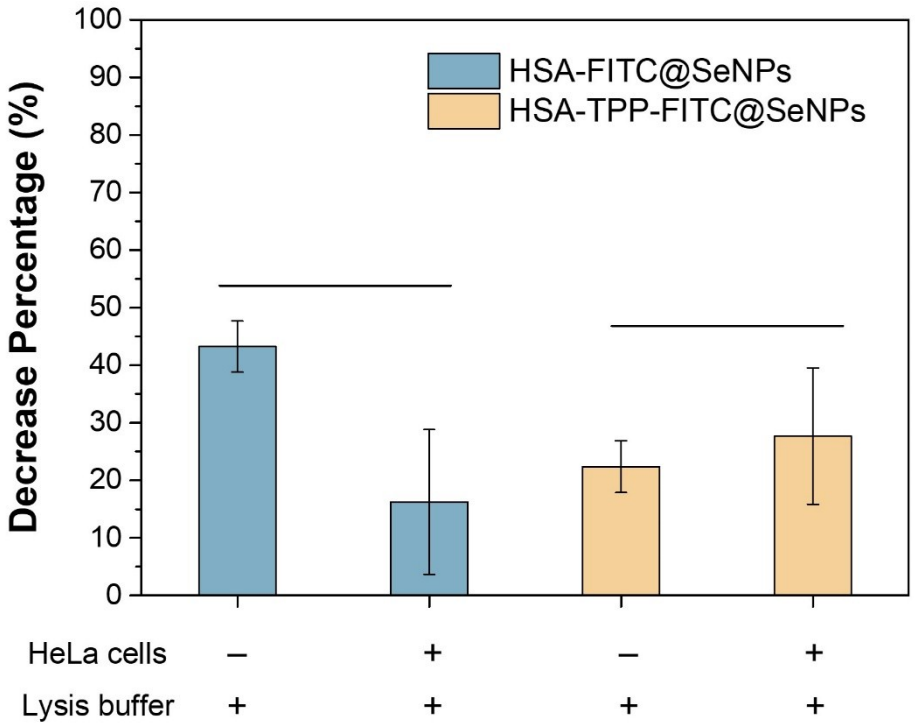


**Figure S12.** Decrease percentage of FITC fluorescent intensity before and after SeNPs purification. Excitation wavelength: 492 nm; emission wavelength: 525 nm. Error bar indicates standard deviation (SD) of triplicate tests. $p > 0.15$ (left) & $p > 0.68$, no significant difference.

**2.7 Colocalization ratio between SeNPs and mitochondria dye**

Cell images were taken by Olympus FV1200 laser scanning confocal microscope (LSCM), and analyzed by ImageJ software (Version 1.52). For a series of cell images including green channel (SeNPs) and red channel (mitochondria), integrated fluorescent intensity of only green channel was measured as $F_g$. Then the merge channel was construct by overlaying the green channel and the red channel. Integrated fluorescent intensity of this merge channel was then measured as $F_m$. Value $R_{g/m}$ indicates ratio of mitochondria-targeted SeNPs among all SeNPs inside cell.

$R_{g/m} = F_g / F_m \times 100\ \%$

# 3 References

1 S. Chakrabortty, B. K. Agrawalla, A. Stumper, N. M. Veg, S. Fischer, C. Reichardt, M. Kogler, B. Dietzek, M. Feuring-Buske, C. Buske, S. Rau and T. Weil, *J. Am. Chem. Soc.*, 2017, **139**, 2512-2519.