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Supplementary Information

The conjugation of rhodamine B enables carrier-free mitochondrial delivery of functional proteins

Jiayuan Shi[1], Dan Zhao[1], Xiang Li[1], Feng Ding[1], Xuemei Tang[1], Nian Liu[2], Hua Huang[1], Changlin Liu[1,*]

[1]School of Chemistry, Central China Normal University, Wuhan 430079, Hubei, China.

[2]School of Basic Medical Sciences, Wuhan University, Wuhan 430071, Hubei, China.

*Corresponding author. Email: liuchl@mail.ccnu.edu.cn (C. L. L)

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TRANSPARENT METHODS

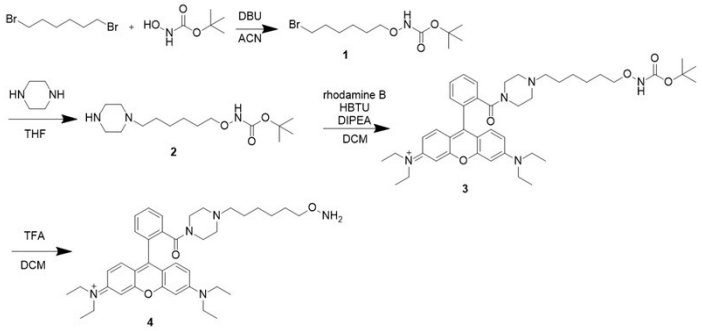
Reagents

All reagents were purchased from commercial suppliers (e.g., Sigma-Aldrich, Energy Chemical, and Roche) and used without further purification unless stated otherwise. Albumin from human serum (lyophilized powder, 96%-99%), cytochrome c from equine heart (lyophilized powder, ≥95%), copper-zinc superoxide dismutase from bovine erythrocytes (lyophilized powder, ≥3000 units/mg protein), catalase from bovine heart (2000-5000 units/mg protein), myoglobin from equine heart (salt-free, lyophilize powder, ≥90%), hemoglobin from bovine blood (lyophilized powder), Human recombinant insulin (lyophilized powder, ≥25 units/mg protein), ovalbumin from chicken egg (lyophilized powder, ≥98%) were also purchased from commercial sources (e.g., Sigma-Aldrich). HRP-conjugated Goat anti-Rabbit IgG (H+L) polyclonal antibody (Cat# ab6721; RRID:AB_955447), Rabbit monoclonal anti-Superoxide Dismutase 1 for WB (Cat# ab51254; RRID:AB_882757) and Rabbit monoclonal anti-Superoxide Dismutase 1 for IP (Cat# ab16831; RRID:AB_302535) were purchased from Abcam.

General instrumentation and sample analyses

Absorption spectra were recorded on a Specord 210 Ultra-visible spectrophotometer (Analytic Jena AG, GER). Fluorescence spectra were performed on a Cary Eclipse fluorescence spectrophotometer (Varian, USA). Circular dichroism (CD) spectra were performed using a Chariscan spectrometer (Applied Photophsics, USA), and all spectra were recorded at 25 ^{o}C. ^{1}H spectra were measured on a Varian Mercury 400 spectrometer. LC/MS/MS was obtained on API 2000 (Applied Biosystems, USA). MALDI-TOF-MS was performed on a Synapt G2 system (Waters, USA). Samples used for MALDI-TOF-MS were co-crystallized with α-cyano-4-hydroxycinnamic acid (CHCA) in 1:1 acetonitrile (MeCN)/H_2O with 0.1% trifluoroacetic acid (TFA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or Native-PAGE was carried out on an electrophoresis Power Pac Basic Power Supply (Bio-Red, USA) using a 12%-15% linear gradient polyacrylamide gel. H&E sections were examined on an inverted fluorescence microscope (Olympus, JPN)

1. Synthesis of N-RhB



Scheme S1. The synthetic route of N-RhB

*1-Bromo-6-(N-tert-butoxycarbonyl-aminooxy)-hexane (**1**).* To a 50 mL round-bottomed flask was equipped with tert-butyl-N-hydroxycarbamate (2.00g, 15mmol) dissolved in

acetonitrile (10 mL). 1, 8-diazabivyclo[5.4.0]undec-7-ene (DBU) (2.23 mL, 15.0 mmol) was added into this solution. Then, the mixture was added to a solution of 1, 6- dibromohexane (4.61mL, 30.0mmol) dissolved in 5 mL acetonitrile slowly. After 3 h of stirring at room temperature, an additional 1 mL of DBU was added. After another 2 h of stirring at room temperature, the reaction temperature was increased to 50 ^{o}C and was stirred overnight at this temperature. The mixture was concentrated under reduced pressure and the residue was dissolved in 9: 1 ethyl acetate: methanol (50mL) and filtered through diatomite to eliminate DUB·HBr salt. The filtrate was concentrated under reduced pressure. The product was purified by SiO_2 gel column chromatography. The column was washed with a gradient from 0-20% ethyl acetate in hexanes, yielding **1** (1.63g, 37%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.49 (s, 1H), 3.86 (t, J = 6.5 Hz, 2H), 3.42 (t, J = 6.8 Hz, 2H), 1.88 (p, J = 6.7 Hz, 2H), 1.66 (p, J = 6.6 Hz, 2H), 1.52 – 1.41 (m, 13H); MS: 297. 3 $[M+H]^+$.

*Tert-butyl(6-(piperazin-1-yl) hexyl)oxycarbamate (**2**).* To a 50 mL round-bottomed flask was added piperazine (4.57 g, 53 mmol) in tetrahydrofuran (15 mL). The solution was heated to reflux and then **1** (1.58 g, 5.3 mmol) was added over 30 minutes. The reaction was stirred at reflux for 4 h. The mixture was filtered and the filtrate was concentrated under reduced pressure. The crude oil was partitioned between water and saturated NaCl and aqueous layer was extracted with water for 3 times. The organic layers were combined and dried over sodium sulfate, filtered and concentrated under reduced pressure to yield **2** (0.60 g, 37%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 3.82 (t, J = 6.6 Hz, 2H), 2.88 (t, J = 4.8 Hz, 4H), 2.40 (s, 4H), 2.32 – 2.27 (m, 2H), 1.65 – 1.58 (m, 2H), 1.45 (s, 11H), 1.43 – 1.24 (m, 6H); MS: 302. 3 $[M+H]^+$.

*Rhodamine B 4-(6-N-tert-butoxycarbonyl-aminooxy-hexyl)piperazine amide (**3**).* To a 50 mL round-bottomed flask rhodamine B (0.60 g, 1.25 mmol) and O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluorophosphate (HBTU) (0.54 g, 4.00 mmol) were added and then the mixture was dissolved in dichloromethane (20 mL). To this solution N,N-diisopropylethylamine (DIPEA) (0.4 mL, 2.3 mmol) was added. After stirring for 30 minutes, compound 2 (0.57 g, 1.89 mmol) was added. The reaction was stirred at room temperature for 8 h, and then quenched with 0.5 M aqueous HCl. The aqueous was separated and extracted with dichloromethane for three times. The combined organic layers were dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude material was further purified by flash chromatography to give **3** (0.78 g, 57%) as a metallic dark purple solid. ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 7.78 – 7.71 (m, 2H), 7.67 – 7.61 (m, 1H), 7.49 (dd, J = 5.7, 3.1 Hz, 1H), 7.24 (d, J = 9.5 Hz, 2H), 7.05 (dd, J = 9.5, 2.4 Hz, 2H), 6.95 (d, J = 2.4 Hz, 2H), 3.70 (dq, J = 21.3, 6.8 Hz, 12H), 3.41 (s, 2H), 3.29 (q, J = 1.6 Hz, 6H), 2.36 (s, 2H), 1.61 – 1.55 (m, 2H), 1.45 (s, 13H), 1.31 (t, J = 7.1 Hz, 14H); MS: 727. 5 $[M+H]^+$.

*Rhodamine B 4-(6-aminooxy-hexyl)piperazine amide (**4, N-RhB**).* To a 25 mL round-bottomed flask was added 3 (100 mg, 0.138 mmol) in dry dichloromethane (5 mL). To this solution trifluoroacetic acid (TFA) (5 mL) was added at room temperature and this solution was left without stirring for 30 minutes. The solvent was then removed under reduced pressure. dichloromethane (5 mL) was added and the solvent was again evaporated under reduced pressure and this procedure was repeated for three times to yield **4** (84 mg, 97%) as a metallic dark purple solid. ^{1}H NMR (400 MHz, CD_3OD) δ 7.78 – 7.76 (m, 1H), 7.76 –

7.71 (m, 2H), 7.50 (dd, J = 5.7, 3.0 Hz, 1H), 7.22 (d, J = 9.5 Hz, 2H), 7.05 (dd, J = 9.5, 2.3 Hz, 2H), 6.94 (d, J = 2.2 Hz, 2H), 4.01 (t, J = 6.3 Hz, 2H), 3.78 – 3.54 (m, 12H), 3.29 (dt, J = 3.2, 1.6 Hz, 6H), 1.76 – 1.66 (m, 4H), 1.46 – 1.39 (m, 4H), 1.31 (t, J = 7.0 Hz, 14H); MS: 627.4 $[M+H]^+$.

2. A general procedure for the modification of commercial proteins

Protein-N-RhB. Pyridoxyl 5'-phosphate (PLP) (20 μmol, final concentration 10 mM) was added to a solution of commercial proteins (100 nmol, final concentration 50 μM) in 25 mM phosphate buffer at pH 6.5. The resulting solution was stirred overnight at 37 °C and then concentrated via ultracentrifugation (Ultracel® centrifugal filter 10000 MWCO, or Ultracel® centrifugal filter 3000 MWCO for purification of modified insulin). The PLP was further removed from the concentrated solution via HiTrap™5 column chromatography (GE Healthcare, eluting into ddH_2O). To this purified solution, **4** (10 μmol, final concentration 2.5 mM) was added and the mixture was allowed to be stirred overnight at room temperature[1]. The reaction mixture was concentrated and further purified by the method described above to yield N-RhB specifically modified proteins named protein-N-RhB. After lyophilization, protein powders were obtained. ***SOD1-RhB.*** To a solution of rhodamine B (1 μmol, final concentration 1 mM) in 50 mM PBS at pH 7.4, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) (5 μmol, final concentration 5 mM), and N-hydroxysuccinimide (NHS) (2 μmol, final concentration 2 mM) were added. This mixture was stirred for 4 h at 4 °C. To this solution was added SOD1 (100 nmol, final concentration 100 μM) and reaction mixture was allowed to be stirred overnight at room temperature[2]. For smaller volume, small molecules were removed from the reaction mixture via ultracentrifugation (Ultracel® centrifugal filter 10000 MWCO) and the mixture was further purified by HiTrap™5 column chromatography (GE Healthcare, eluting into ddH_2O) to yield RhB nonspecifically modified SOD1 named SOD1-RhB. After lyophilization, protein powders were obtained. ***SOD1-FITC.*** SOD1 (100 nmol, final concentration 100 μM) in 50 mM carbonate buffer solution was added fluorescein isothiocyanate (FITC) (1 μmol, final concentration 1 mM). The reaction mixture was allowed to be stirred for 2 h at room temperature. To remove free FITC, the solution was concentrated via ultracentrifugation (Ultracel® centrifugal filter 10000 MWCO) and further purified by HiTrap™5 column chromatography (GE Healthcare, eluting into ddH_2O) to yield FITC nonspecifically modified SOD1 named SOD1-FITC. After lyophilization, protein powders were obtained. ***FITC-protein-N-RhB.*** SOD1 or HSA (100 nmol, final concentration 100 μM) in 50 mM carbonate buffer solution was added fluorescein isothiocyanate (FITC) (1 μmol, final concentration 1 mM). The reaction mixture was allowed to be stirred for 2 h at room temperature. To remove free FITC, the solution was concentrated via ultracentrifugation (Ultracel® centrifugal filter 10000 MWCO) and further purified by HiTrap™5 column chromatography (GE Healthcare, eluting into ddH_2O) to yield FITC-protein-N-RhB.

3. Cell culture

All Cell lines were obtained from China Center for Type Culture Collection (CCTCC). Cell lines were cultured at 37 °C with a saturating humidity atmosphere containing 5% CO_2.

A549 cells were cultured in 1640 media supplemented with 1% penicillin/streptomycin, and 10% FBS. HEK 293, DU145, HeLa and COS-7 were cultured in DMEM media supplemented with 1% penicillin/streptomycin, and 10% FBS. MRC-5 was cultured in MEM media supplemented with 1% penicillin/streptomycin, and 10% FBS. RWPE-1 was cultured in Defined Keratinocyte-SFM media supplemented with 1% penicillin/streptomycin, and 2‰ Defined Keratinocyte-SFM Growth Supplement. Media, FBS and penicillin/streptomycin were all purchased from Gibco.

4. Assay of self-delivery of the modified proteins

Cells were seeded in 6-well plates at a density of 10^4 cells per mL in corresponding culture media. After 24 h, cells were washed once with PBS and treated with 5 μM protein-N-RhB diluted in serum-free media for 6 h at 37 °C. After being washed three times with PBS containing 20 U/mL heparin (unless specially noted, PBS used in the later experiments were PBS containing 20 U/mL heparin) to remove surface-adhered protein-N-RhB, cells were dissociated by 0.25% trypsin-EDTA and resuspended in PBS. Fluorescence data were collected by flow cytometry (BD, Accuri™ C6) and analyzed by Flowjo 7.6 software. Following the treatment with 5 μM protein-N-RhB, cells were also imaged on an inverted fluorescence microscope (Olympus, IX71) or a laser scanning confocal microscope (Leica, TCS SP8) after washing. Signals were analyzed using filters for rhodamine B emission.

For effects of concentrations, transfection time and temperature on the self-delivery of protein, experiments were carried out according to the following steps. Cells were incubated with 0, 0.5, 1.5, 2.0, and 5 μM SOD1-N-RhB diluted in corresponding media and imaged with an invert fluorescence microscope 6 h after the treatment, respectively. Following the addition of 5 μM SOD1-N-RhB diluted in the 1640 media, A549 cells were imaged 1.5, 3, 6, 10, and 12 h after the treatment with an invert fluorescence microscope, and also imaged 6 h after the addition of 5 μM SOD1-N-RhB at 4 °C and 37 °C, respectively.

For the efflux of protein-N-RhB, A549 cells treated with 5 μM SOD1-N-RhB for 6 h were washed three times with PBS and imaged 0.5, 1.5, 3, 5, 8 h following the replacement of the culture medium with the protein-N-RhB-free fresh one.

All samples were washed three times with PBS before imaging.

5. Assays of SOD1 activity

The activity of SOD1 modified with different small molecules and total activity of SOD1 of A549 cells treated with SOD1-N-RhB were measured with a HT Superoxide Dismutase Assay Kit (Trevigen). Superoxide radical ($O2^{\cdot -}$) generated from the conversion of xanthine to uric acid and H_2O_2 by xanthine oxidase convert WST-1 to WST-1 formazan which absorbs light at 450 nm. SOD1 activity was determined by measuring percent inhibition of the rate of formation of WST-1 formazan[3,4] following the manufacturer's instructions. For the measurements of total activity of intracellular SOD1, samples were lysates of A549 cells treated with varied concentrations of SOD1-N-RhB. The activity of wild-type SOD1 and the total activity of SOD1 in A549 cell lysates treated without SOD1-N-RhB was set as

100% and the results represent the mean ± SD of at least three parallel experiments.

6. Assays of catalase activity

The activity of catalase modified with N-RhB (CAT-N-RhB) was determined respectively using a UV-vis spectrophotometer and a Catalase Assay Kit (Beyotime). The former method was used to measure the activity of pure catalase in solution while the latter one was universally applicable (catalase diluted in solution or in cell lysates). H_2O_2 exhibits a strong UV absorption at 240 nm. Catalase activity was determined by measuring the change of UV absorption at 240 nm within 2 min along with the catalyzed decomposition of H_2O_2 (initial concentration 100 mM)[5]. Furthermore, Catalase Assay Kit was adopted to measure the total activity of intracellular catalase for the reason that many substances in cell lysate show strong absorption at 240 nm. Under the condition of abundant H_2O_2, a portion of which was catalyzed by catalase while the remnant was catalyzed by peroxidase in the Kit contributing to the formation of red product N-(4-antipyryl)-3-chloro-5-sulfonate-p-benzoquinonamonoimine that absorbs light at 520 nm. Catalase activity was calculated from the standard curve made by utilizing the relationship between concentrations of remnant H_2O_2 and UV absorption at 520 nm[6]. Concentrations of H_2O_2 used in the experiments were determined by measuring the UV absorption at 240 nm before use and the spontaneous decomposition of H_2O_2 within 2 min has been confirmed to have little effects on the catalyzed decomposition of H_2O_2 in the presence of catalase or peroxidase. One unit activity was defined as the catalyzed decomposition of 1 μM of H_2O_2 per min at pH 7.4 at 25 ^oC. The activity of wild-type catalase or the total activity of catalase in cells treated without CAT-N-RhB set as 100% and the results represent the mean ± SD of at least three parallel experiments.

For the measurements of total catalase activities in mice organs and tumor tissues, organs and tumor tissues were first sliced and cracked. The total proteins concentrations of cell lysates were measured with a Pierce™ BCA Protein Assay Kit (Thermo Scientific). These samples were diluted to the same concentrations of total proteins. The amounts of total proteins used in liver samples were one-third of others according to the instructions for the reason that catalase contents in liver are much higher[7,8], and catalase activities in these samples were measured by the method described above. The total activity of catalase to catalyze completely 15 nmol of H_2O_2 according to the instructions set as 100% and the results represent the mean ± SD of at least three parallel experiments.

7. Assays of resistance of the modified proteins to proteases

Wild-type SOD1 and SOD1-N-RhB were incubated in the pH 7.4 trypsin solution and the pH 2.0 pepsin solution for 5 h at 37 ^oC, respectively. The activity of SOD1 and SOD1-N-RhB was measured with a HT Superoxide Dismutase Assay Kit (Trevigen) described above after 0.5, 1.5, 3, and 5 h, respectively. Each sample was determined in triplicates. The protease solution (final concentration 10 mg/mL) was prepared according to the following steps. 10 g of trypsin was dissolved in 1 L PBS at pH 7.4 to give the trypsin solution. 10 g of pepsin was dissolved in 1 L ddH_2O, and this solution was adjusted to pH 2 with dilute hydrochloric acid to give the pepsin solution.

8. Assays of stability of the modified proteins

HSA-N-RhB was respectively incubated with PBS, 1640 media, A549 cell lysates and serum for 5 days. Then, one portion of the mixtures was subject to SDS-PAGE, and the remaining mixtures were monitored using AKTA Purifier 10 (HPLC) to check the stability of HSA-N-RhB.

9. Western-blotting

A549 cells were incubated with varied concentrations of SOD1-N-RhB at 37 ^{o}C for 6 h. Cells were lysed using lysis buffer (Trevigen) containing 10 mM of PMSF protease inhibitor (Thermo Scientific) at 4 ^{o}C for 30 min before washing three times with PBS. The cell lysates were then transferred into microfuge tubes and centrifuged at 12,000×g for 10 min at 4 ^{o}C. The supernatant was collected and total protein concentrations were measured with a Pierce^{TM} BCA Protein Assay Kit (Thermo Scientific). The cell lysates with same protein concentrations were mixed with SDS- PAGE loading buffer and then heated to 100 ^{o}C for 10 min. All samples and protein ladder (Thermo Scientific) were loaded to a 12% SDS-PAGE gel and separated by electrophoresis. The proteins on the gel were transferred onto a 0.45 μm PVDF membrane (Thermo Scientific) pre-activated in methanol. Membranes were blocked in 5% BSA diluted in the TBST for 1 hour, and incubated with primary antibody (Abcam) diluted in TBST (1: 5000) overnight at 4 ^{o}C. After washing with TBST for three times to remove free primary antibody, membranes were incubated with goat anti-rabbit-HRP secondary antibody in 1: 2000 dilution for 2 h. Membranes were washed with three times with TBST, strained with a Pierce^{TM} DAB Substrate Kit (Thermo Scientific), and imaged using a GeneGenome Chemiluminescence Imager (Syngene).

10. Immunoprecipitation (IP) assays of intracellular SOD1-N-RhB

A549 cells were cultured in T25 cell culture flask for 24 h, Then media was aspirated and cells were washed once with PBS. For the following treatment with 5 μM SOD1-N-RhB diluted in the serum-free 1640 media for 6 h at 37 ^{o}C. Cells were washed three times with PBS and lysed using the lysis buffer (Trevigen) containing 10 mM of PMSF protease inhibitor (Thermo Scientific) for 30 min at 4 ^{o}C. The total protein concentrations of cell lysates were determined with a Pierce^{TM} BCA Protein Assay Kit (Thermo Scientific). The samples of cell lysates with same protein concentrations were incubated with a primary antibody (Abcam) specific to SOD1 overnight at 4 ^{o}C. 40 μL of well-distributed Pierce^{TM} Protein A+G Agarose beads (Thermo Scientific) was added to the solution and the mixture was allowed to be shaken slowly on an orbital shaker for 2 h. Following centrifugation at 2500 rpm for 5 min, the supernatant was discarded. The precipitate was added 1 mL PBS, centrifuged and the supernatant was discarded. This step was repeated several times. A portion of these Protein A+G Agarose beads in the precipitate was resuspended in PBS and imaged with an inverted fluorescence microscope (Leica, DMI3000B). Protein A+G Agarose beads alone and the sample of Protein A+G Agarose beads incubated with SOD1-N-RhB in the absence of primary antibody dealt with same steps served as control.

11. Assays of intracellular ovalbumin (OVA)

A549 cells were washed three times with PBS after incubation with varied concentrations

of OVA-N-RhB. Cells were lysed and the supernatant of cell lysate was collected following the procedure described above. OVA-N-RhB in the supernatant was measured with a Chicken Ovalbumin (OVA) ELISA Kit (Abebio) following the manufacturer's instructions. Total protein concentrations were measured with a Pierce™ BCA Protein Assay Kit (Thermo Scientific). Standards and samples were added respectively to the microelisa stripplate wells pre-coated with an antibody specific to OVA. Then a HRP-conjugated antibody specific for OVA was added to each well and incubated. Following a wash to remove free components, the TMB substrate solution was added to each well. After the addition of the stop solution, the optical density (OD) was measured with a Spectramax M5 (Molecular Devices, USA) at the wavelength of 450 nm. The concentrations of OVA in the samples were proportional to the OD value and calculated by comparing OD data of samples to the standard curve. The concentrations of OVA in samples represent as ug OVA/mg total proteins.

12. RT-qPCR

A549 cells were washed three times with PBS 6 h after incubation with varied concentrations of SOD1-N-RhB. Total RNA was extracted with a High Pure RNA Isolation Kit (Roche). Purified RNA (1 μg) was reversely transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche). The mRNA levels were monitored a LightCycler® 96 (Roche) with FastStart Essential DNA Green Master (Roche). Correct amplification was monitored via post-run melt curve analysis, and the quantity of mRNA was calculated by delta delta Ct method[9].

RT-qPCR primers for SOD1 and β-actin:

SOD1: 5'-AAGGCCGTGTGCGTGCTGAA-3'; 5'-GGCCCACCGTGTTTTCTGGA-3'

β-actin: 5'-CCACACCTTCTACAATGAGC-3'; 5'-TGAGGTAGTCAGTCAGGTCC-3'

13. Measurements of intracellular H_2O_2 levels

A549 cells were incubated with varied concentrations of SOD1-N-RhB or CAT-N-RhB diluted in serum-free 1640 media for 6 h, and then dichloro-dihydro-fluoresceindiacetate (DCFH-DA) (Sigma) was added to the media at a final concentration of 5 μM for the measurements of intracellular H_2O_2[10-12]. After incubation for 20 min, A549 cells were washed three times with PBS to remove non-internalized protein-N-RhB and dyes. A549 cells were dissociated by 0.25% trypsin-EDTA and resuspended in PBS. Fluorescence intensity of cells was analyzed using a flow cytometry (BD, Accuri™ C6). Fluorescence of SOD1-N-RhB and CAT-N-RhB had been confirmed to have little interference on the detection of dyes before the experiments.

14. Assays of subcellular localization

A549 cells were incubated with SOD1-N-RhB diluted in serum-free 1640 media for 6 h. 0.1μg/mL of Hoechst 33342 (MedChemExpress), 0.1 μM of MitoTracker® Green (Invitrogen) or 0.1 μM LysoTracker® Green (Invitrogen) was respectively added to the media and incubated for 30 min. Following a wash with PBS for three times, cells were imaged with a laser scanning confocal microscope (Leica, TCS SP8) using corresponding filters for respective dyes and images were analyze using the LAS-AF-Lite software. To

further show the co-localization of protein conjugates with mitochondria, FITC-SOD1-N-RhB was first obtained by modifying SOD1-N-RhB with FITC and it was also able to enter into cells analyzed by flow cytometry. A549 cells were incubated with FITC-SOD1-N-RhB in serum-free 1640 media for 6 h. Then, 0.1 μM of MitoTracker® Blue was added and incubated for another 30 min. Following washing, cells were imaged with LSCM. The uptake, motion and efflux of SOD1-N-RhB were performed by real-time imaging with a LSCM. Cells were first treated with SOD1-N-RhB and imaged in 1, 2, 3, 5, 7, 9, 13, 15, 20, 25, and 30 min, respectively. For the dynamic colocalization of SOD1-N-RhB within mitochondria or lysosome, cells were respectively treated with 0.1 μM of MitoTracker® Green (Invitrogen) and 0.1 μM LysoTracker® Green (Invitrogen) for 30 min prior to the addition of SOD1-pRhB, and imaged in 3, 10 and 30 min with a LSCM.

15. Assays of the uptake mechanism

A549 cells were pretreated with the specific inhibitors (chlorpromazine, nystatin, nocodazole, chloroquine, cytochalasin D, and amiloride hydrochloride) of different endocytosis processes, sodium chlorate or specific substrates of OCTs and novel OCTs for 1 h at 37 ^{o}C. Then, A549 cells were incubated with SOD1-N-RhB for another 6 h. Following a wash with PBS for 3 times, A549 cells were analyzed with a flow cytometry or imaged with an inverted fluorescence microscope. To explore the effect of free N-RhB in competing the uptake of protein conjugates, FITC-SOD1-N-RhB was first obtained by modifying SOD1-N-RhB with FITC and FITC-SOD1-N-RhB was pre-verified to enter into cells by flow cytometry. A549 cells were pretreated with free N-RhB and incubated with FITC-SOD1-N-RhB for another 6 h. Following washing, fluorescence data of FITC was collected with a flow cytometry.

16. Toxicity assays

MTT assay was used to determine the cytotoxicity of protein-N-RhBs. A549 cells were incubated with varied concentrations of wild-type proteins or protein-N-RhBs diluted in serum-free media. Following the incubation for 12 h at 37 ^{o}C, media were removed and cells were washed three times with PBS, and cells were then treated with 0.5 mg/mL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) (Sigma) diluted in 200 μL serum-free 1640 media for another 4 h at 37 ^{o}C. Media were aspirated and 150 μL DMSO was added to the plates to dissolve MTT formazan crystals. Following the oscillation to make MTT formazan crystals be dissolved fully, the optical density (OD) of each plate was recorded with a Spectramax M5 (Molecular Devices, USA) at the wavelength of 490 nm. Cell viability (%) was present as mean of OD of samples/ mean of OD of cells incubated without proteins×100.

In vivo toxic examinations on normal mice of CAT-N-RhB were performed on Balb/c mouse. 15 normal mice were randomly divided into three groups (5 mice each group) and administered respectively by intravenous injection of saline as well as the modified and WT CAT (doses: 10 mg/kg/2 days, 20 days). The mouse weight was measured and recorded every other day for 20 days.

17. Assays of *in vivo* distribution of SOD1-N-RhB

In vivo fluorescence imaging of Balb/c mouse organs was performed at WHU (Wuhan University) on an Xtreme Imaging System (Bruker, GER). 4-week-old female mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China) and all experiments were carried out according to the Chinese Regulations for the Administration of Affairs Concerning Experimental Animals. 100 μL of SOD1-N-RhB (150 μM) or N-RhB (150 μM) diluted in PBS was injected intravenously into mice. 2, 4, 6, 8, and 24 h after injection, all mice were dissected, and major organs (brain, heart, spleen, lung and kidney) were collected[13]. The fluorescence signals of these organs were recorded on Xtreme Imaging System at an exposure time of 1.2 s for all images. The final images were present as overlaid images with fluorescent and X-ray images.

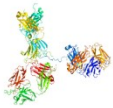
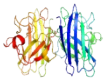
18. *In vivo* antitumor study of CAT-N-RhB

All animals studies were guided by the regulations set by Institutional Animal Care and Use Committee and project protocols were approved by the Animal Ethics Committee of Huazhong Agricultural University. Female Balb/c-nude mice (4-6 weeks) were bought from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). To set up the A549 tumor-bearing mice model, 5×10^6 A549 cells were injected subcutaneously into the right axillary space of mice. When tumor volumes reached around 70 mm^3, the tumor-bearing mice were randomly divided into 3 groups (5 mice per group), named “Saline”, “CAT”, and “CAT-N-RhB”, respectively. These three groups of A549 tumor-bearing mice were intravenously injected every other day with saline, CAT (10 mg/kg), and CAT-N-RhB (10 mg/kg), respectively. The mouse weight and tumor sizes were measured and recorded every other day for 22 days. Tumor sizes were measured by a vernier caliper and tumor volume was calculated using the following formula: $V = a\times b^2/2$, where a and b were the longest and shortest diameters of tumors, respectively. At last, all mice were sacrificed and tumors as well main organs were excised on day 22. Tumors were weighed and used for subsequent experiments. Tumors and other main organs (kidney) were collected and fixed in 10% neutral formalin solution, and then, paraffin embedded sectioning was conducted for histological hematoxylin and eosin (H&E) staining. The pathological sections were examined with a microscope (Olympus, IX71).

19. Statistical Analysis

Quantitative data are presented as mean ± standard error and statistical significance for absolute or relative values were calculated using Student’s t test in Microsoft Excel. The p values to controls were marked with asterisks, *p< 0.05, **p< 0.01, ***p< 0.001.

Table S1. Proteins selected for the self-delivery.

Proteins	Source	Molecular weight	Structures	N- terminal amino acids	PDB ID	Crystal structures	Functions	Medicinal uses
Catalase (CAT)	Bovine	247 kDa	Tetramer 32% α-helix 17% β-sheet	Asn	5KGN		Catalyzes the decomposition of H_2O_2	Textile bleaching/scavenger of H_2O_2 in food industry/antimicrobial agents; Biochemical research for cancer
Cytochrome c (Cyt C)	Equine	12 kDa	Monomer 40% α-helix 1% β-sheet	Gly	1HRC		Plays a role in apotosis	First-aid medicine for hypoxia
Hemoglobin (Hb)	Bovine	64.5 kDa	Tetramer 75% α-helix (α chain) 82% α-helix (β chain)	Val (α chain) Met (β chain)	2QSS		Transports molecular oxygens	Biochemical research
Human serum albumin (HSA)	Human	67 kDa	Dimer 70% α-helix	Ser	1AO6		Regulates colloidal osmotic pressure of blood/transport hormones, fatty acids, drugs	Replace lost fluid and help restore blood volume/reverse toxicity of drugs
Insulin (Ins)	Human Recombinant	5.8 kDa	Hexamer 46% α-helix, 10% β-sheet (A chain) 57% α-helix, 9% β-sheet (B chain)	Gly (A chain) Phe (B chain)	5E7W		Treats high blood glucose	Treatment for diabetes
Immunoglobulin G (IgG)	Human	150 KDa	Monomer 8% α-helix, 44% β-sheet (heavy chain) 6% α-helix, 49% β-sheet (light chain)	Gln (heavy chain) Glu (light chain)	1HZH		Participates in humoral immunity; binds and neutralizes toxins; plays an important role in antibody-dependent cell-mediated cytotoxicity. e.g.	Prevention of measles and infectious hepatitis; Prevention of bacterial and viral infections
Myoglobin (Mb)	Equine	17 kDa	Monomer 73% α-helix	Gly	1YMB		Stores molecule oxygen	Marker for myocardial damage
Ovalbumin (OVA)	Chicken	42.7 kDa	Dimer 31% α-helix, 32% β-sheet	Gly	1UHG		A storage protein	Immunization drug
RNase A (RNase)	bovine	13.7 KDa	Monomer 20% α-helix, 35% β-sheet	Lys	3A1R		Catalyzes the degradation of RNA	Treatments for cancer and hepatitis B; Inhibition of replication of HIV-1 virus
Cu,Zn-superoxide dismutas (SOD1)	Bovine	32 kDa	Dimer 4% α-helix, 41% β-sheet	Ala	1CHJ		Catalyzes the conversion of $O_2^{\cdot-}$ to O_2 and H_2O_2	Treatment for cardiovascular disease, autoimmune disease. e.g.

SUPPLEMENTAL FIGURES

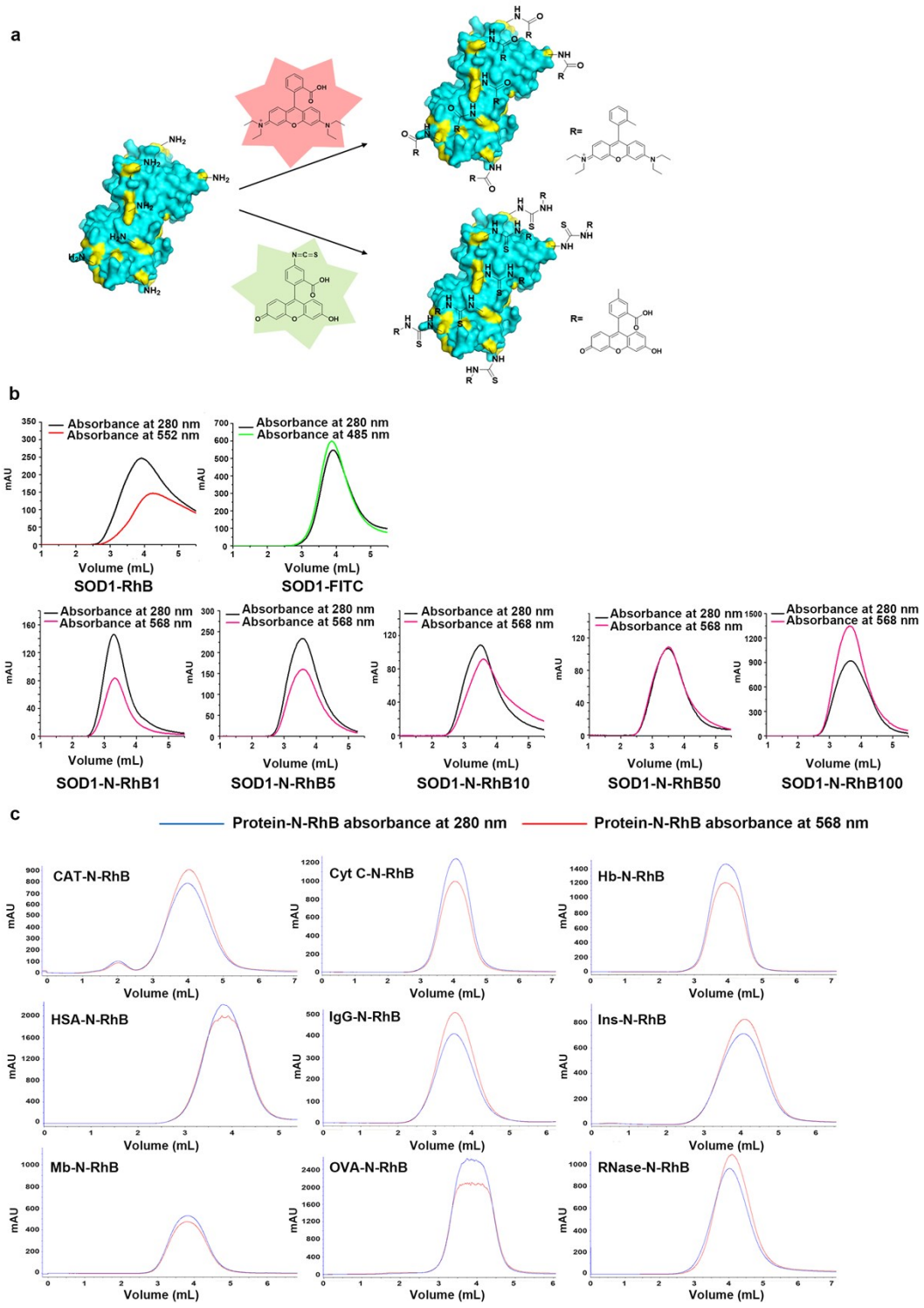


Fig. S1 The scheme of non-specific modification and UV-vis traces of Protein-N-RhBs during purification. (a) SOD1 were nonspecifically modified with either RhB (SOD1-RhB) or FITC (fluorescein isothiocyanate, SOD1-FITC). (b) UV-vis traces of SOD1-RhB, SOD1-FITC and SOD1-N-RhB (the reaction molar ratios of N-RhB and SOD1 are 1, 5, 10, 50, and 100, respectively). (c) UV-vis traces of the other protein-N-RhBs during purification.

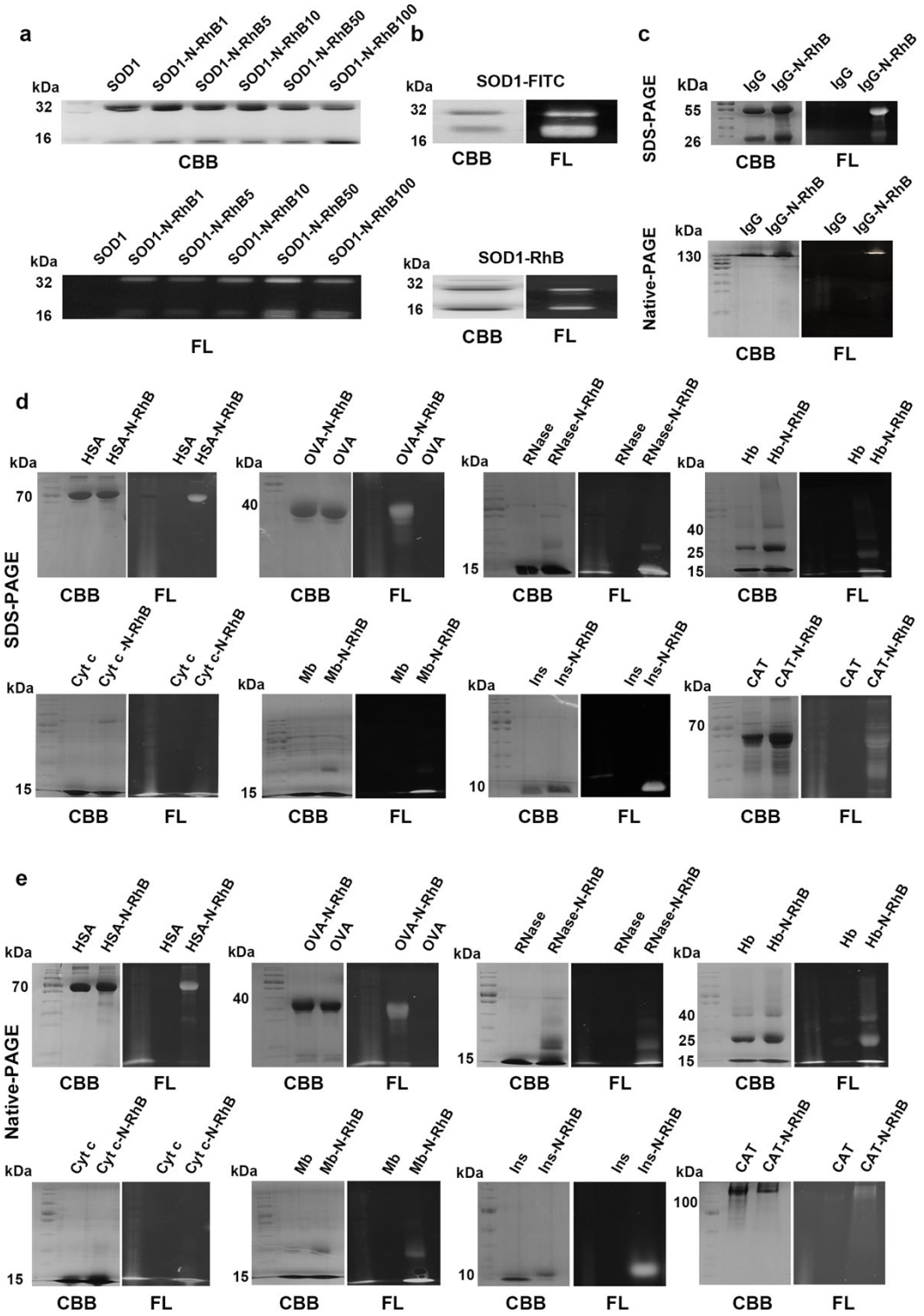


Fig. S2 PAGE analysis of the modified proteins. (a) Native-PAGE of SOD1 modified with increasing molar ratio of N-RhB (SOD1-N-RhB1, 5, 10, 50, and 100 stands for the reaction molar ratios of N-RhB to SOD1: 1, 5, 10, 50, and 100, respectively). (b) Native-PAGE of SOD1-FITC and SOD1-RhB. (c) SDS-PAGE and Native-PAGE of IgG-N-RhB. (d) SDS-PAGE of protein-N-RhBs. (e) Native-PAGE of protein-N-RhBs. CBB and FL, indicating Coomassie Brilliant Blue staining and in-gel fluorescent scanning, respectively.

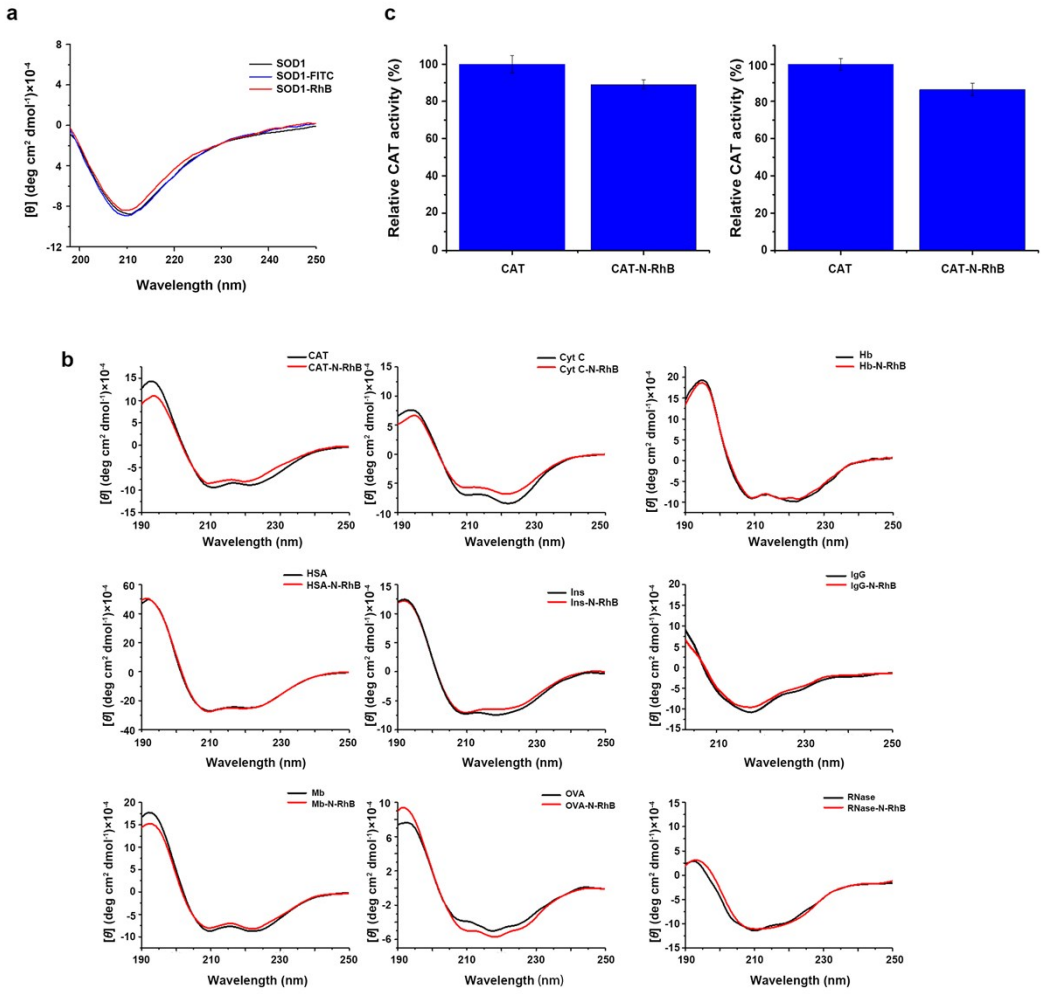


Fig. S3 Circular dichroism (CD) and activity measurements of the modified proteins. (a) CD measurements of non-specifically modified SOD1. These modification of RhB or FITC did not alter the structure of SOD1. (b) CD measurements of protein-N-RhBs. CAT-N-RhB, Cyt C-N-RhB, Hb-N-RhB, HSA-N-RhB, Ins-N-RhB, IgG-N-RhB, Mb-N-RhB, OVA-N-RhB and RNase-N-RhB exhibited no significant structure changes towards Native proteins. (c) Activity measurements of catalase. Activity of wild-type CAT and CAT-N-RhB was measured by a commercial kit (left) and UV-Vis absorption spectra (right). After the specific N-terminal modification, CAT still maintained the activity. Error bars indicate ± SD from triplicates of independent experiments.

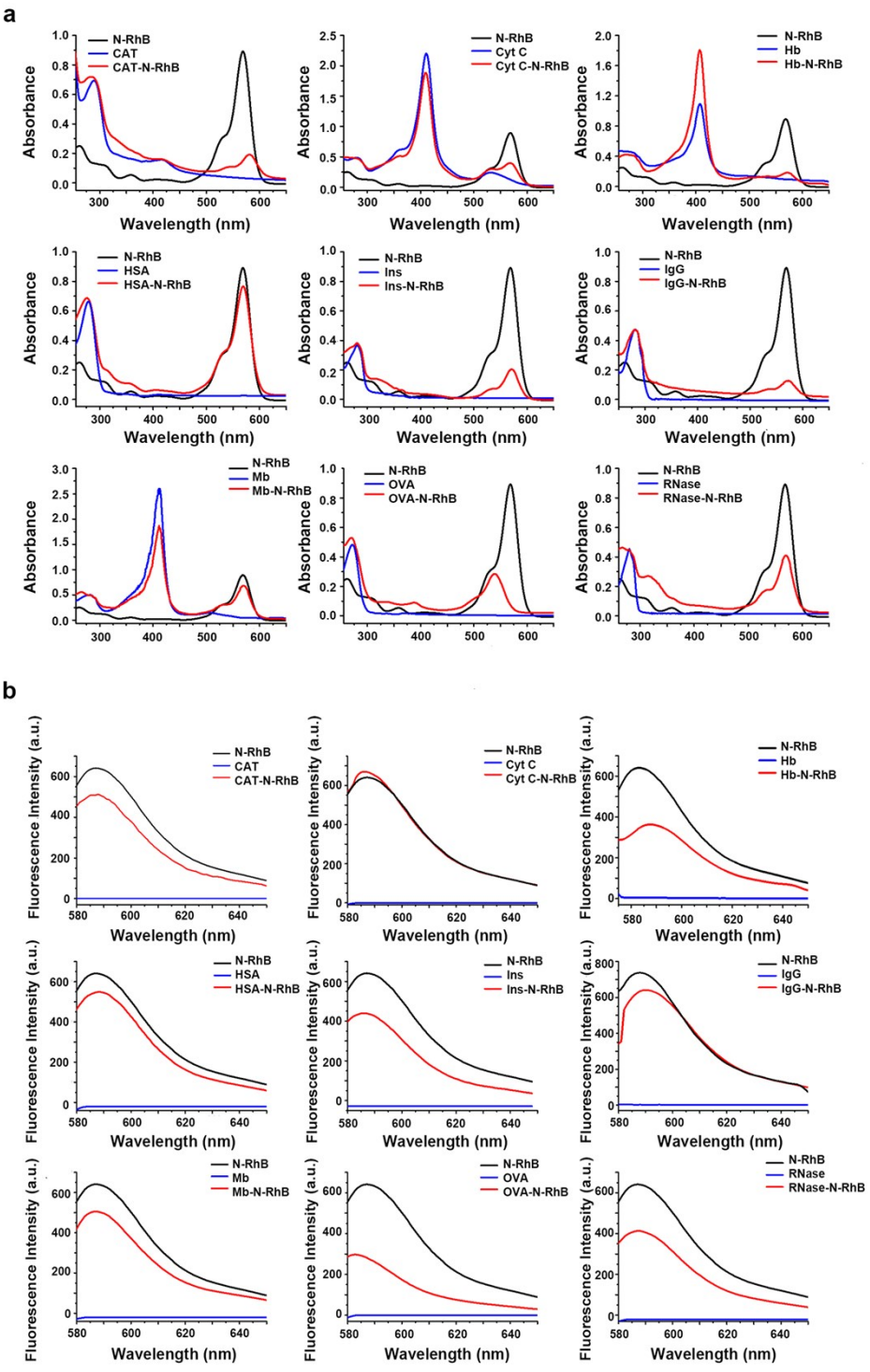


Fig. S4 Ultraviolet-visible and fluorescence spectroscopy of Protein-N-RhBs. (a) UV-Vis absorption and (b) fluorescence spectra of CAT-N-RhB, Cyt C-N-RhB, Hb-N-RhB, HSA-N-RhB, Ins-N-RhB, IgG-N-RhB, Mb-N-RhB, OVA-N-RhB and RNase-N-RhB.

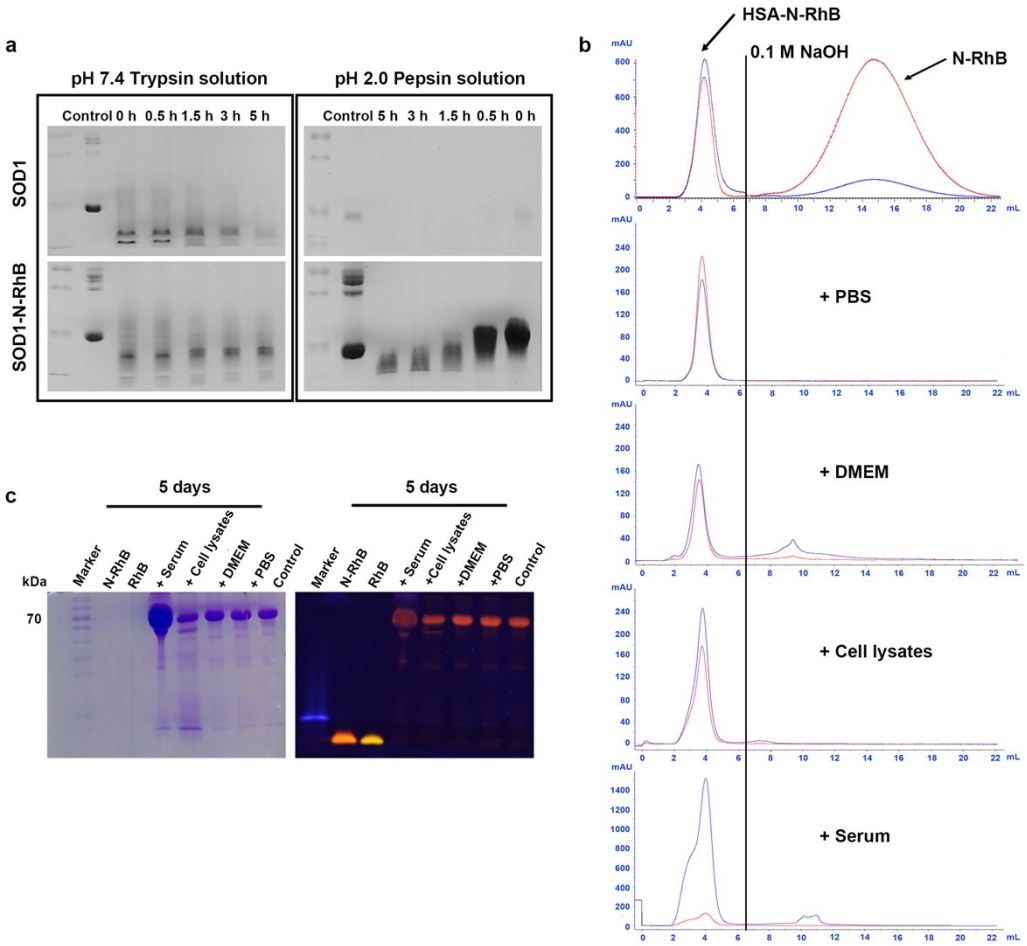


Fig. S5 The stability analysis of protein-N-RhB. (a) The resistance of SOD1 and SOD1-N-RhB to proteases. SDS-PAGE of SOD1 and SOD1-N-RhB, respectively, in pH 7.4 Trypsin solution and in pH 2.0 Pepsin solution. (b) UV-vis traces during purification of HSA-N-RhB treated with PBS, 1640 media, cell lysates and serum, respectively, at 37 ^{o}C for 5 days. (c) SDS-PAGE of HSA-N-RhB treated with PBS, 1640 media, cell lysates and serum, respectively, at 37 ^{o}C for 5 days.

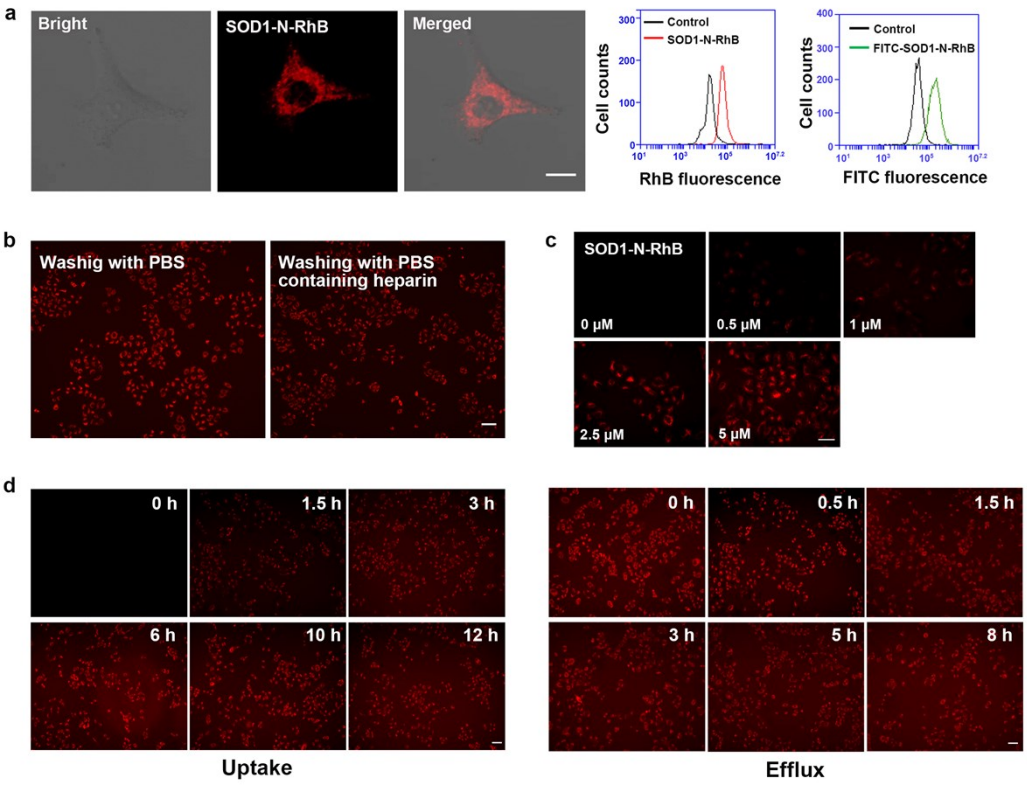


Fig. S6 The uptake and efflux of SOD1-N-RhB by A549 cells. (a) Self-delivery of SOD1-N-RhB and FITC-SOD1-N-RhB was observed respectively by LSCM and flow cytometry (Scale bars, 10 μm). (b) Effects of heparin on the self-delivery of SOD1-N-RhB into A549 cells. To rule out the effect of the modified proteins bound on cell surfaces, cells were washed three times with PBS containing 20 U/mL heparin, removing the non-internalized SOD1-N-RhB. (c) Concentration-dependent uptake of SOD1-N-RhB. Elevated delivery of SOD1-N-RhB into A549 cells with increasing concentrations. (d) Time-dependent uptake and efflux of SOD1-N-RhB. SOD1-N-RhB reached its highest concentration in cytoplasm for 6 h incubation and was partially excreted out of cells (Scale bars, 50 μm).

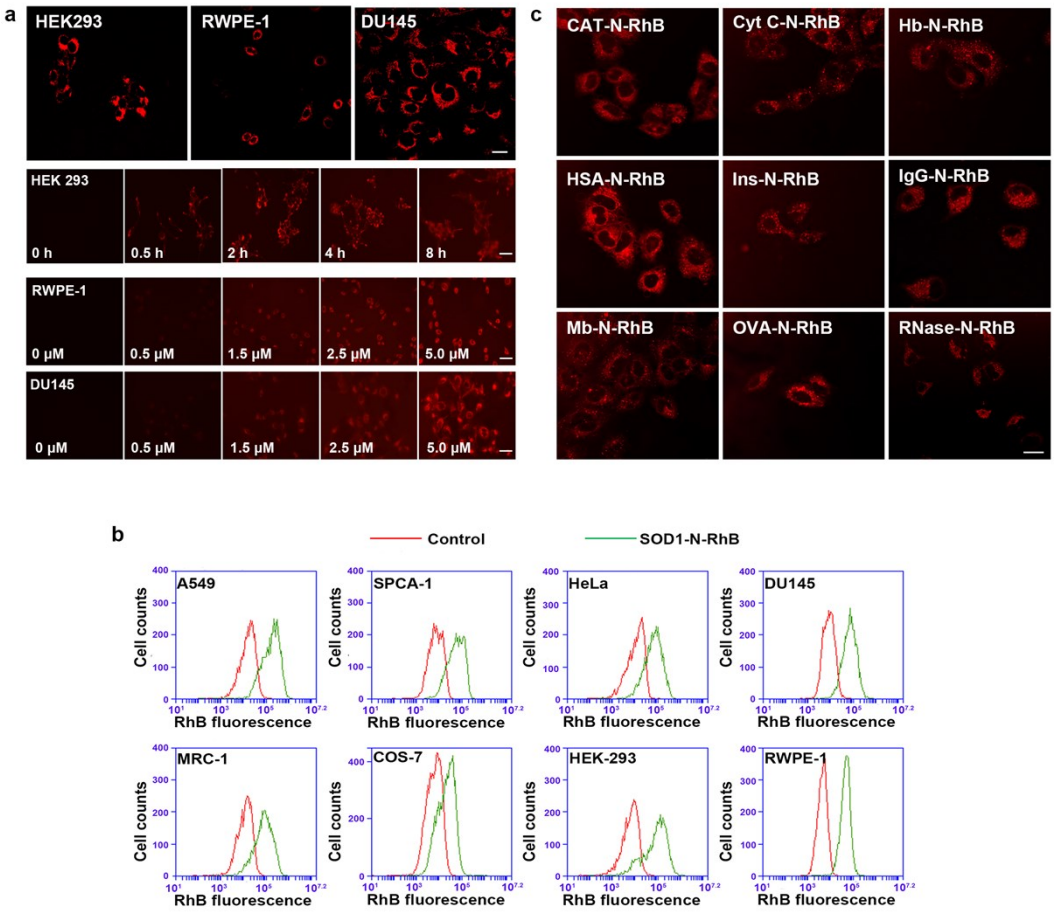


Fig. S7 Self-delivery of Protein-N-RhBs into different cell types. (a) Uptake of SOD1-N-RhB by HEK293, RWPE-1 and DU145. SOD1-N-RhB entered into cells in a time-dependent and concentrations-dependent manner. (b) Successful uptake of SOD1-N-RhB by eight cell types measured by flow cytometry. (c) Self-delivery of Protein-N-RhBs into A549 cells. Another nine modified proteins were observed to enter into A549 cells under LSCM (scale bars, 20 μm).

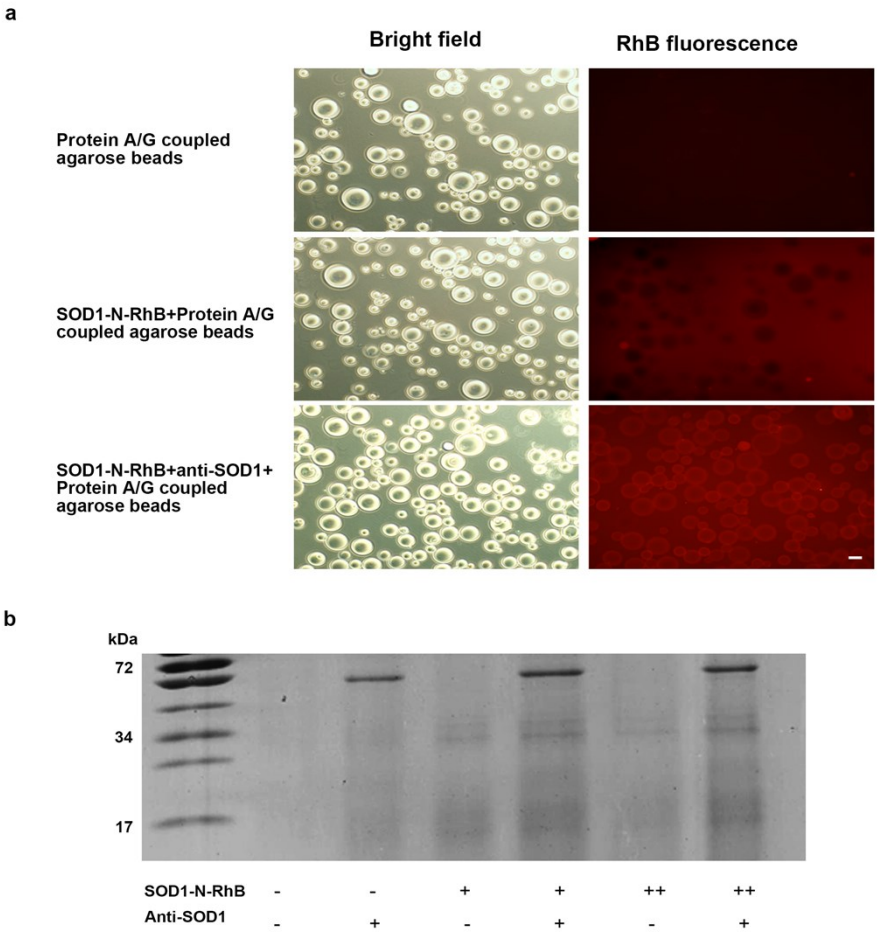


Fig. S8 Detection of SOD1-N-RhB via immunoprecipitation (IP). (a) SOD1-N-RhB immunoprecipitated by SOD1 antibody could be captured by Protein A/G coupled agarose beads through bead surface-bound anti-SOD1 antibody in dilute solutions. (scale bars, 20 μm). (b) SDS-PAGE analysis of SOD1-N-RhB from the cellular SOD1-loaded beads. The contents of intracellular SOD1 were observed to increase with the elevated concentrations of SOD1-N-RhB.

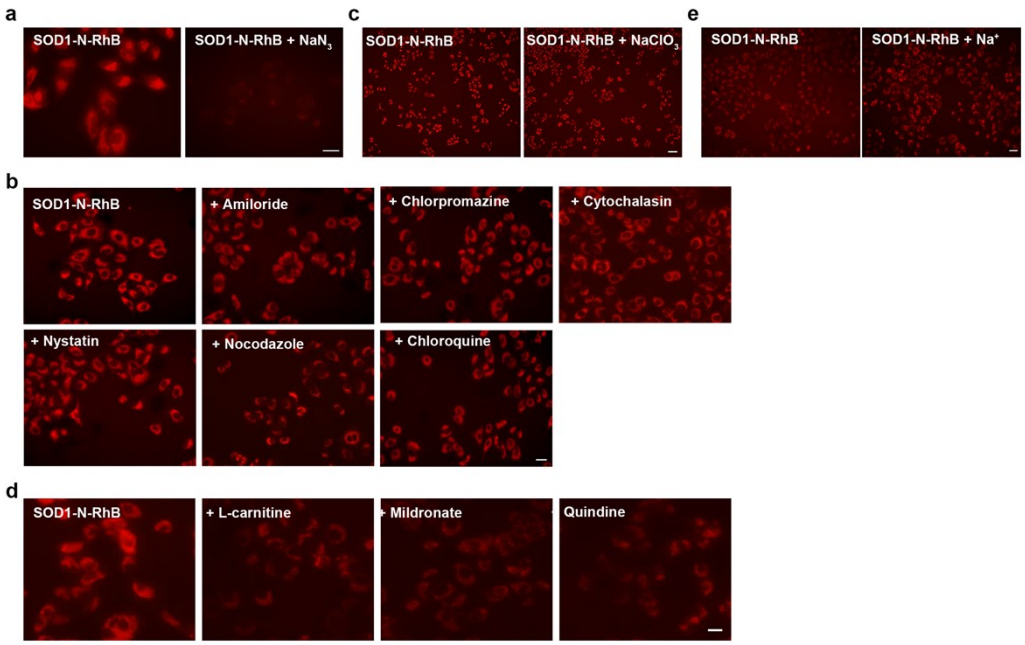


Fig. S9 Effects of inhibitors on the uptake of SOD1-N-RhB. (a) Effects of NaN_3 on the uptake of SOD1-N-RhB. Cellular uptake of SOD1-N-RhB was blocked at 37 ^oC by NaN_3, a general inhibitor of active transportation. (b) Effects of endocytosis inhibitors on the uptake of SOD1-N-RhB. The specific endocytosis inhibitors have minimal effects on the self-delivery of SOD1-N-RhB. (c) Effects of $NaClO_3$ on the uptake of SOD1-N-RhB. Self-delivery of SOD1-N-RhB into A549 cells was not blocked by $NaClO_3$. (d) Effects of substrates or inhibitors of OCTs/OCTNs on the uptake of SOD1-N-RhB. Self-delivery of protein-N-RhB into A549 cells was blocked by inhibitors of OCTs/OCTNs. (e) Effects of Na^+ on the uptake of SOD1-N-RhB. Self-delivery of SOD1-N-RhB into A549 cells was influenced by the incubation in the culture media with or without Na^+ (scale bars, 20 μm).

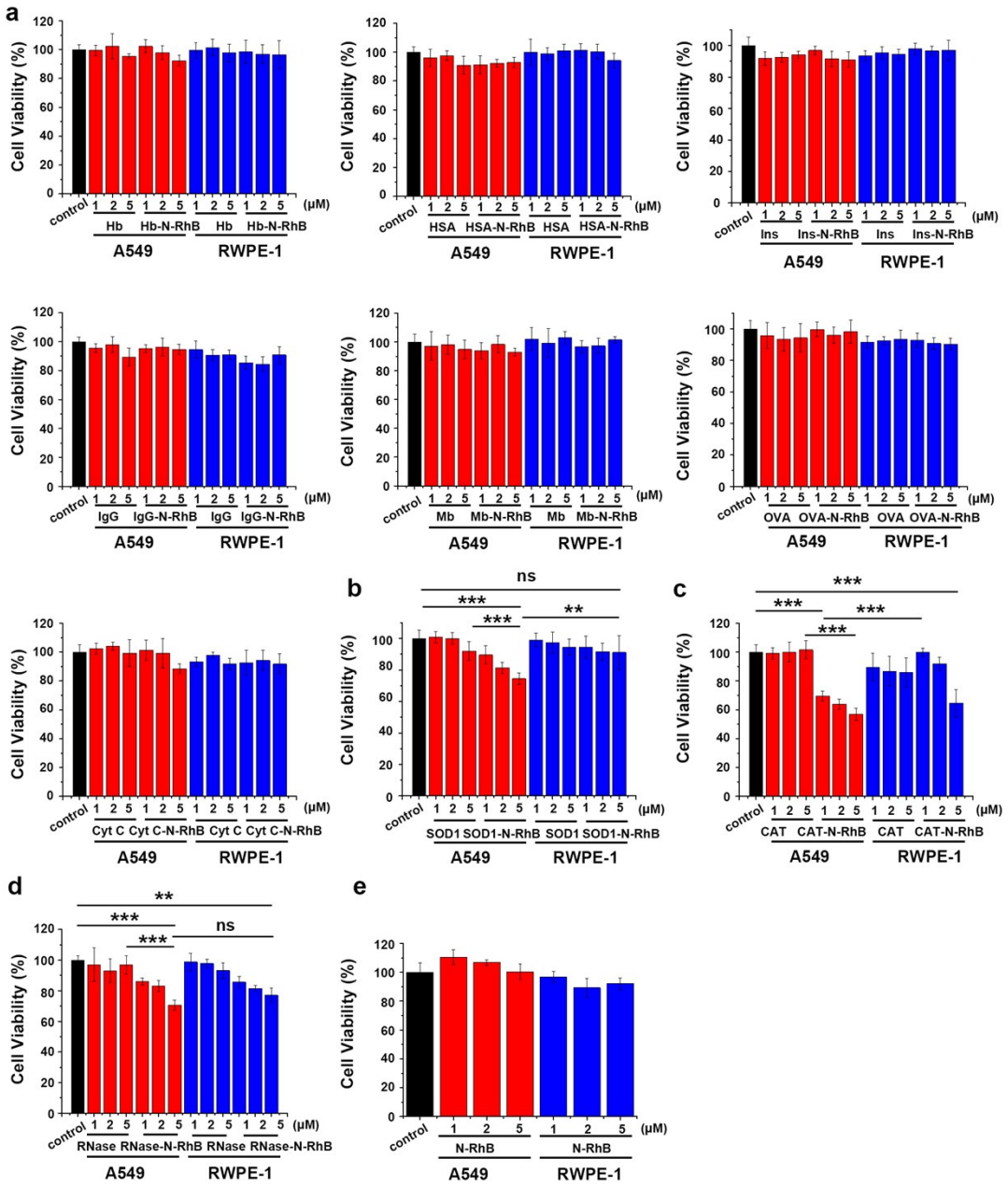
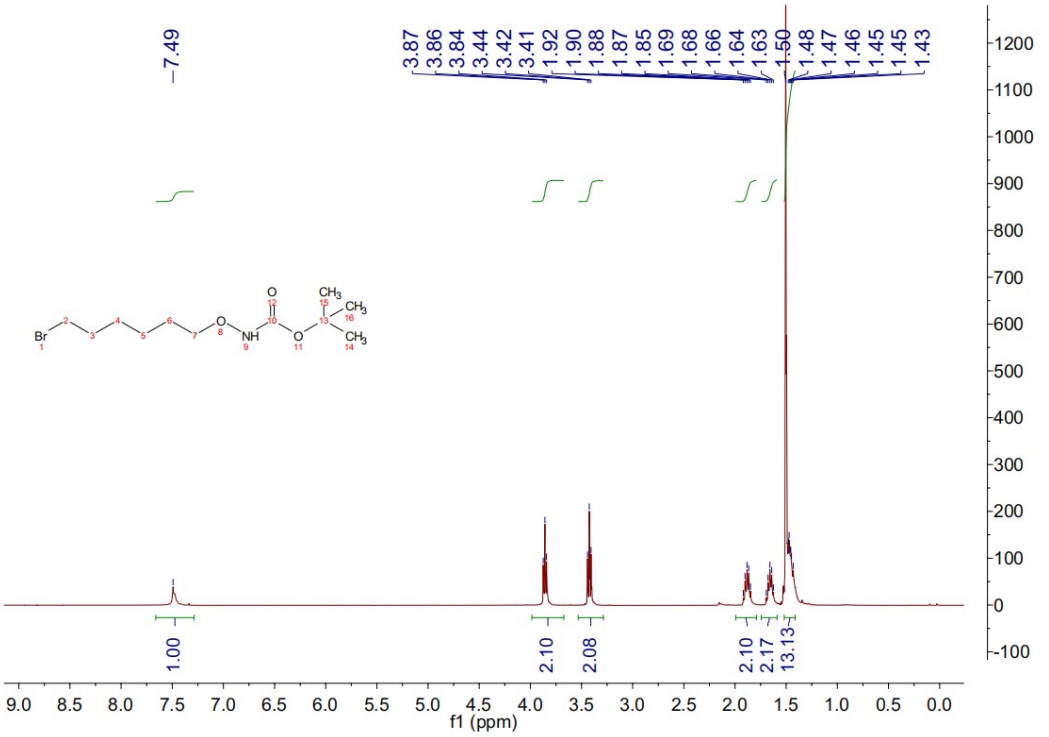
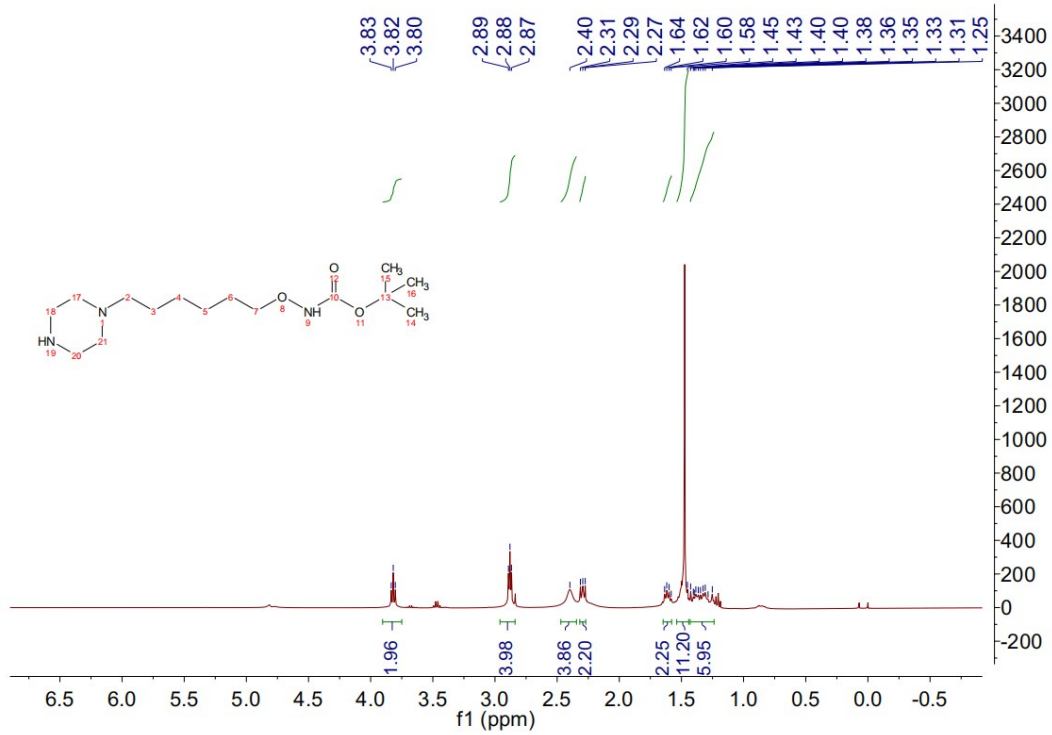
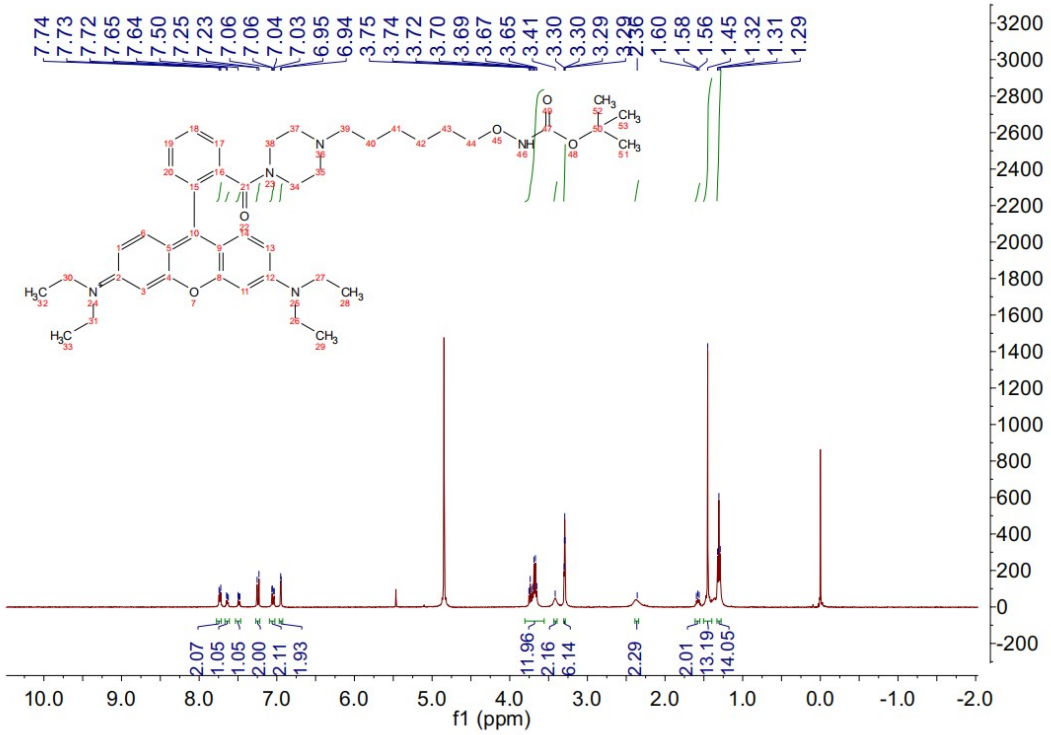


Fig. S10 Toxicity of the modified proteins on normal and cancer cells. Viability of A549 (cancer) and RWPE-1 (normal) cells respectively exposed to the modified Hb, HSA, Ins, IgG, Mb, OVA, Cyt C (a), SOD1 (b), CAT (c), RNase (d) and N-RhB (e) measured by MTT assay.

^{1}H-NMR spectra of compounds **1**-**4**



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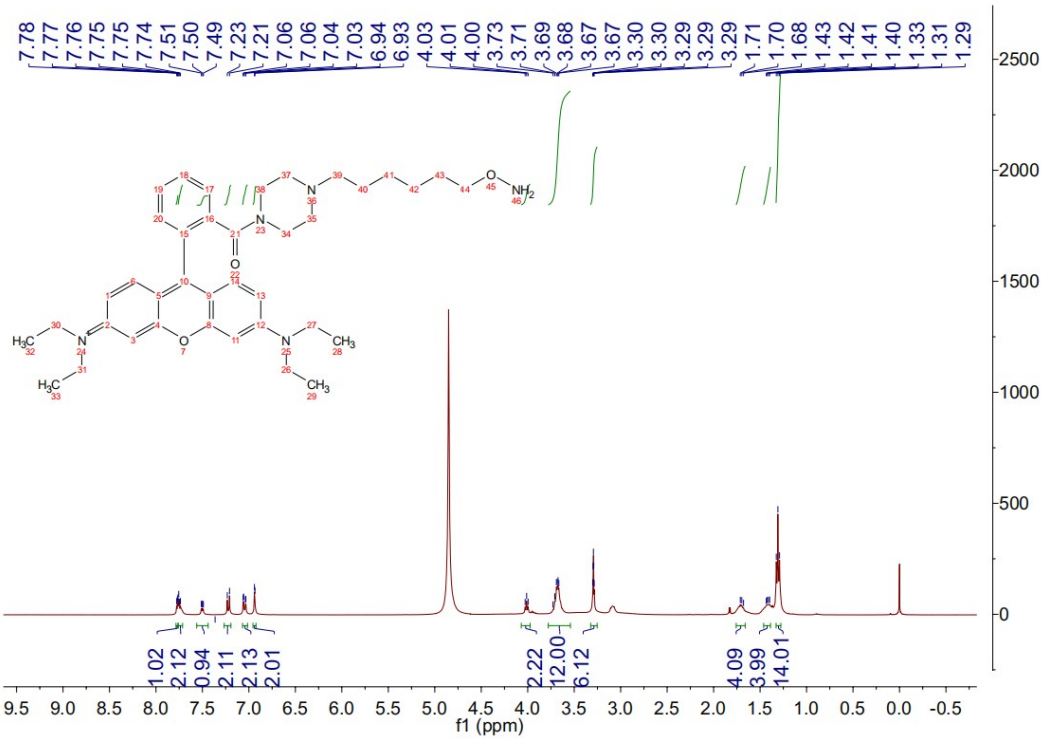
2.07 1.05 1.05 2.00 2.11 1.93 11.96 2.16 6.14 2.29 2.01 13.19 14.05
f1 (ppm)


7.78 7.77 7.76 7.75 7.75 7.74 7.51 7.50 7.49 7.23 7.21 7.06 7.06 7.04 7.03 6.94 6.93 4.03 4.01 4.00 3.73 3.71 3.69 3.68 3.67 3.30 3.30 3.29 3.29 3.29 1.71 1.70 1.68 1.43 1.42 1.41 1.40 1.33 1.31 1.29
1.02 2.12 0.94 2.11 2.13 2.01 2.22 12.00 6.12 4.09 3.99 14.01
f1 (ppm)

REFERENCE

1. Gilmore, J. M.; Scheck, R. A.; Esser-Kahn, A. P.; Joshi, N. S.; Francis, M. B. *Angew. Chem. Int. Ed.* **2006,** 45, 5307-5311.
2. Rosenbaugh, E. G.; Roat, J. W.; Gao, L.; Yang, R. F.; Manickam, D. S.; Yin, J. X.; Schultz, H. D.; Bronich, T. K.; Batrakova, E. V.; Kabanov, A. V.; Zucker, I. H.; Zimmerman, M. C. *Biomaterials* **2010,** 31, 5218-5226.
3. Peskin, A. V.; Winterbourn, C. C. *Clin. Chim. Acta.* **2010,** 293, 157-166.
4. Tan, A. S.; Berridge, M. V. *J. Immunol. Methods* **2010,** 238, 59-68.
5. Beers, R. F. Jr.; Sizer, I. W. *J. Biol. Chem.* **1952,** *195,* 133-140.
6. Fan, J.; Yin, J. J.; Ning, B.; Wu, X.; Hu, Y.; Ferrari, M.; Anderson, G. J.; Wei, J.; Zhao, Y.; Nie, G. *Biomaterials* **2011,** *32*, 1611-1618.
7. Deisseroth, A.; Dounce, A. L. *Physiol, Rev.* ***1970,*** *50,* 319-375.
8. Schisler, N. J.; Singh, S. M. *Genome* **1987,** 29, 748-760.
9. Livak, K. J.; Schmittgen, T. D. *Methods* **2001,** 25, 402-408.
10. Juarez, J. C.; Manuia, M.; Burnett, M. E.; Betancourt, O.; Boivin, B.; Shaw, D. E.; Tonks, N. K.; Mazar, A. P.; Doñate, F. *Proc. Natl. Acad. Sci. USA* **2008,** 105, 7147-7152.
11. Fujimura, M.; Morita-Fujimura, Y.; Kawase, M.; Copin, J. C.; Calagui, B.; Epstein, C. J.; Chan, P. H. *J. Neurosci.* **1999,** *19,* 3414-3422.
12. Bindokas, V. P.; Jordán, J.; Lee, C. C.; Miller, R. J. *J. Neurosci.* **1996,** 16, 1324-1336.
13. Sun, Y.; Ding, M.; Zeng, X.; Xiao, Y.; Wu, H.; Zhou, H.; Ding, B.; Qu, C.; Hou, W.; Er-Bu, A.; Zhang, Y.; Cheng, Z.; Hong, X. *Chem. Sci.* **2017,** 8, 3489-3493.