Electronic Supplementary Material (ESI)

Change in the structure and function of lectin by photodissociation of NO

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1. Experimental procedures

Preparation of metallothionein.

Open reading frame clone encoding the full length of horse metallothionein-1B (MT-1B; accession No. XM_003364597) in the pET-15b vector was purchased from Genscript (USA) and transformed into *E. coli* strain BL-21 codon plus. *E. coli* strains were cultured in LB medium at 37 °C in the presence of ampicillin, and expression of MT was induced with 0.25 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) when the turbidity (OD_{600}) of the suspension was larger than 0.8. Following incubation for 2.5 h, cells were harvested and suspended in the solubilizing buffer (6 M urea, 1 mM dithiothreitol (DTT), 10 mM imidazole, 1 %(w/v) triton, 1%(w/v) streptomycin, and 20 mM Tris-HCl, pH = 8), and incubated for an hour to extract proteins from cells. After centrifuging the mixture at 2200 g for an hour, MT was isolated from the supernatant using a Ni^{2+}-charged medium (Ni Sepharose 6 Fast Flow, GE Healthcare) in a batch mode. The purified sample was dialyzed against 10 mM Tris-HCl buffer (pH=8) containing 0.5 mM DTT for 12 h, distilled water for 24 h, and milli-Q water for 9 h. The obtained sample was lyophilized and stored at -80 °C. Commercially available MT-1B (M4766, Aldrich) was also used in the experiments.

Preparation of WT hGal-1. Recombinant WT hGal-1 was obtained from *E. coli* cells transformed with a plasmid DNA encoding the full amino acid sequence of WT hGal-1.[1] The transformed *E. coli* cells were cultured in LB medium at 37 °C for 6 h in the presence of ampicillin. Expression of WT hGal-1 was induced with 0.1 mM IPTG for 3 h.[2,3] The cells were harvested and disrupted with a sonicator (Sonifier250, Branson) in 20 mM phosphate buffer (pH 7.5) containing 150 mM NaCl, 4 mM β-mercaptoethanol (β-ME), 2 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride (a protease inhibitor). Nucleic acids were removed from the cell lysate by centrifugation at 16000 g and 4 °C for 15 min in the presence of 1% (w/v) streptomycin (Nacalai Tesque), and the supernatant was collected. WT hGal-1 was purified from the supernatant using a lactose gel affinity column (EY laboratories). The purified sample was dialyzed against distilled water, lyophilized, and then purified further by gel filtration chromatography (Sephacryl S-100, GE Healthcare) with

the gel running buffer containing 20 mM phosphate (pH 7.5), 150 mM NaCl, and 4 mM β-ME. Collected fractions were again dialyzed against doubly distilled water, and lyophilized and stored at –80 °C.

Preparation of apo-MT. Metal ions were removed from MT prior to the addition reaction of NO. The removal of metal ions was carried out by incubating MT in 100 mM Tris-HCl buffer (pH 9) containing 25 mM DTT and 15 mM EDTA overnight at 37 °C. After the incubation period, the solution was made acidic by adding 1/50-fold of trifluoroacetic acid (TFA), and then purified by NAPTM-25 Column (Amersham Biosciences) with 0.1 % (v/v) TFA as a mobile phase. Powdered apo-MT was obtained by lyophilization and stored at -80 °C. The amount of thiol groups of apo-MT was estimated in advance by the 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) method.

Preparation of SNO-hGal-1 using SNO-MT. Cysteine residues of WT hGal-1 were fully reduced in advance by incubating in 500 μL of 100 mM Tris-HCl buffer solution (pH = 8.3) containing 5 mM DTT for 2 h at 37 °C. Then, the buffer solution was exchanged with 100 mM sodium phosphate buffer (pH 7.0) using the PD-10 desalting column (GE Healthcare). SNO-MT was prepared by adding $NaNO_2$ to 500 μL of apo-MT solution in 100 mM HCl in which the molar ratio of $NaNO_2$ to thiol groups of apo-MT was 2:1, and the mixture was incubated for 2 min on ice in the dark. Then the NO-addition reaction was stopped by adding 90 μL of 1 M Tris-HCl buffer solution (pH 9.3) to 500 μL of the SNO-MT solution. The amount of the generated SNO groups was estimated using the molar coefficient of nitrosoglutathione at 334 nm (767 M cm^{-1}).[4,5] The obtained SNO-MT solution was mixed with reduced WT hGal-1 by stirring, and was incubated for an hour at room temperature in the dark. The molar ratio of SNO groups was prepared to be approximately seven fold of thiol groups of WT hGal-1. The incubated solution was centrifuged at 15000 g for 5 min and the supernatant was collected. SNO-hGal-1 was isolated by the PD-10 desalting column equilibrated by water, and lyophilized in the dark and stored at –80 °C.

Preparation of SNO-hGal-1 using SNO-Cys. Nitrosocysteine (SNO-Cys) was prepared by mixing 70 mg of $NaNO_2$ and 120 mg of cysteine in 5 mL of 0.1 M HCl solution on ice and in the dark. This mixture was three-fold diluted by the same sodium phosphate buffer and the amount of SNO groups was estimated using the molar coefficient at 545 nm (14.9 M cm^{-1}).[6] The reduced WT hGal-1 was prepared using the same method described above. The SNO-Cys solution was added to WT hGal-1 and the molar ratio of WT hGal-1 to SNO-Cys was adjusted to be ca. 1:120. The mixture was incubated for an hour at room temperature in the dark. SNO-hGal-1 was isolated by the PD-10 desalting column using milli-Q water as a mobile phase, lyophilized, and stored at –80 °C.

Photoirradiation experiments. SNO-hGal-1 or WT hGal-1 was dissolved in 100 mM sodium phosphate buffer (pH 7), containing 150 mM NaCl. 500 μL of the sample solution was put in each well of an 8-well chambered coverglass and then it was subjected to light irradiation from the bottom by 10 Hz of the pulsed light from the second or third harmonic generation (532 nm or 355 nm) of Nd:YAG laser (Brilliant, Quantel). The laser power was adjusted to be 6 mJ/pulse and 60 mJ/pulse for 355 nm and 532 nm, respectively. The irradiation times for experiments of optical spectra and an affinity column chromatography were 15 and 45 min for 355 nm and 532 nm light, respectively. When the photoirradiation effect was analysed using an affinity column chromatography, the sample solution was loaded to a lactose affinity column (EY laboratories), followed by washing with 10 mL of the same sodium phosphate buffer to remove unbound hGal-1. The flow-through from the column was pooled, and the absorbance at 220 nm (A_{w220}) was measured. hGal-1 bound to the column was eluted by 10 mL of the same buffer with 100 mM of lactose and the flow-through from the column was again pooled and its absorption spectrum was measured. The contribution of lactose in the spectrum was subtracted and the absorbance at 220 nm coming from bounded hGal-1 (A_{e220}) was obtained. The sugar binding ability was then estimated by $100\times A_{e220}/(A_{w220}+A_{e220})$.

Examination of photoirradiation effect of direct hemagglutination of erythrocytes was carried out as follows. SNO-hGal-1 or WT hGal-1 was dissolved in 10 mM sodium phosphate

buffer (pH 7.4), containing 150 mM NaCl, and the concentration of hGal-1 was adjusted to be 1 μM. Rabbit or sheep blood was ca. 200 fold diluted by the same buffer solution. Equal aliquots of these solutions were mixed and incubated for 30 min. Then, 150 μL of the mixture was put in each well of an 8-well chambered coverglass and then subjected to the light irradiation from the bottom by the output from Nd:YAG laser as mentioned above. The irradiation times were 60 and 90 min for 355 nm and 532 nm light, respectively. The sample solution was gently pipetted before and after the photoirradiation, and then the image of the sample solution was observed using a conventional microscope with a 40X objective lens.

Hemagglutination formed by WT hGal-1 in the presence of SNO-Cys was photoirradiated with the same protocol as that mentioned above except that SNO-Cys was added in the medium to be 3 μM before the photoirradiation. The third harmonic (355 nm) of the output from the Nd:YAG laser was used as the photoirradiation source and the laser power and the irradiation time were 6 mJ/pulse and 60 min, respectively.

Preparation of oxidised WT hGal-1. H_2O_2 was added to be 10 mM into buffer solution including WT hGal-1, and the solution was incubated for 60 min to obtain oxidised WT hGal-1. In hemagglutination assay with oxidised WT hGal-1, sheep blood was ca. 400 fold diluted by the buffer solution, and then 150 μL of the blood solution was put in each well of an 8-well chambered coverglass. Oxidised WT hGal-1 was added into the blood solution to be 0.5 μM and the solution was incubated for 30 min. Then the image of the solution was observed using a conventional microscope with a 40X objective lens. Absorption spectrum of oxidised WT hGal-1 was obtained by the subtraction of absorption spectrum of H_2O_2 from the mixture of oxidised WT hGal-1 and H_2O_2. We also confirmed that the same spectrum was obtained by the removal of H_2O_2 in the mixture using gel filtration chromatography.

DTNB assay after photoirradiation. DTT was added to be ca. 5 mM into the buffer solution including photoirradiated SNO-hGal-1. The solution was incubated for 2 h at 37 °C to perform the

reduction of photoirradiated SNO-hGal-1, and DTT was removed from the solution by gel filtration chromatography. The concentration of hGal-1 was adjusted to be 5 μM using buffer solution, and DTNB was added to be 50 μM, and then the absorption spectra were measured. Buffer solution was degassed before the assay. DTNB assays of the photoirradiated SNO-hGal-1 without the reduction procedure and SNO-hGal-1 without photoirradiation were also performed with the same procedure.

Acquisition of absorption and CD spectra. Absorption spectra of the prepared solutions were recorded in a quartz cell with a 10 mm path length using a Hitachi U-3300 spectrophotometer. The baseline of the absorption spectrum of the photoirradiated SNO-hGal-1 was evaluated by fitting the spectrum in the region of 320–500 nm using a third-order polynomial function. IGOR Pro Ver. 6.37 was used for the fitting procedure. For measuring circular dichroism (CD) spectra, Jasco J-820 spectropolarimeter was used. CD spectra were measured before and after photoirradiation in a quartz cells with a 0.5 mm path length. The spectra of individual samples were averaged over four scans, and the measured ellipticity values were converted to mean residue molar ellipticity.

SDS-PAGE. Separation gel and stacking gel were prepared to be 20 wt% and 4.5 wt% acrylamide, respectively. The mixture of samples and Laemmli sample buffer with or without 2-mercaptoethanol were boiled at 100°C for 3 min before loading. Precipitates were dissolved in Tris-HCl buffer containing 20 mM DTT before the mixing to Laemmli sample buffer. The electric field was applied for 140 min with the constant current mode (20 mA) and the obtained bands were visualized with Coomassie Brilliant Blue dye.

2. Figures

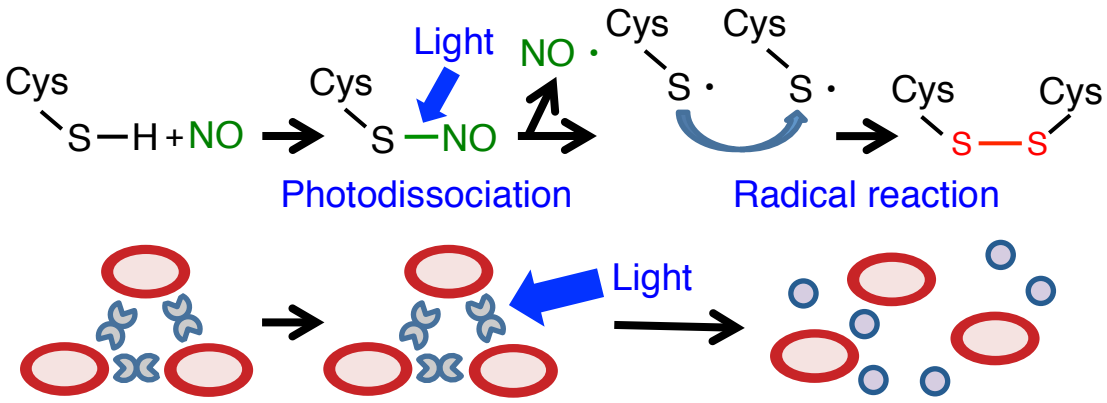

Fig. S1. Schematic diagram of the dissociation of cell aggregation using photodissociation of NO.

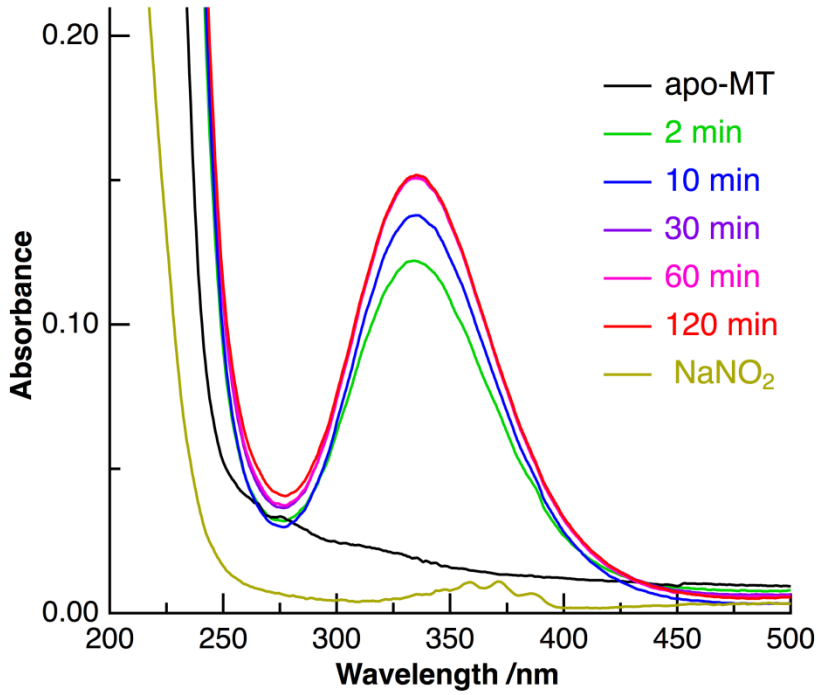

Fig. S2. Absorption spectra of apo-MT-1B at different times after the mixing of $NaNO_2$ under light exclusion at 0°C and pH ~1. Absorption spectrum of apo-MT-1B before the mixing and that of $NaNO_2$ are also shown in the same figure for comparison.

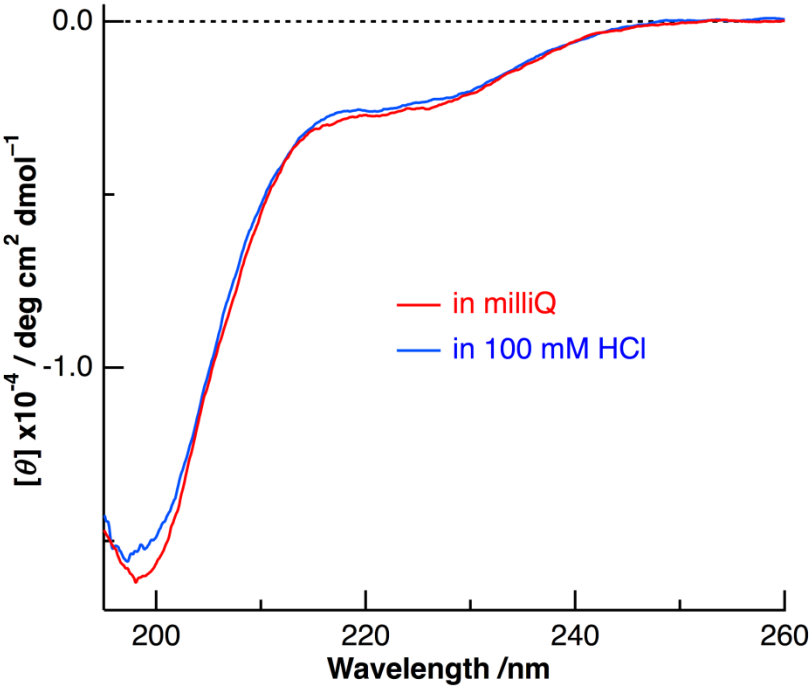

Fig. S3. CD spectra of apo-MT-1B in milliQ (red) and in 100 mM HCl (blue). The concentration of apo-MT-1B was 34 μM.

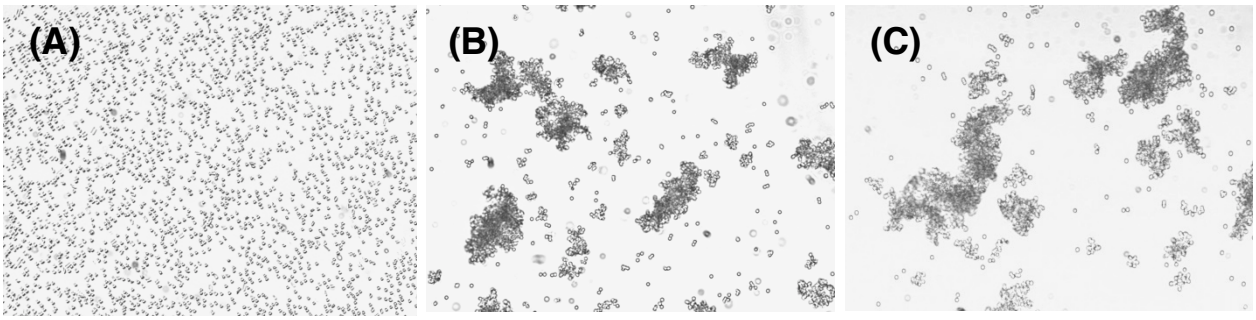

Fig. S4. Representative images of rabbit erythrocytes without any lectin (A), with SNO-hGal-1 (B), and WT hGal-1 (C).

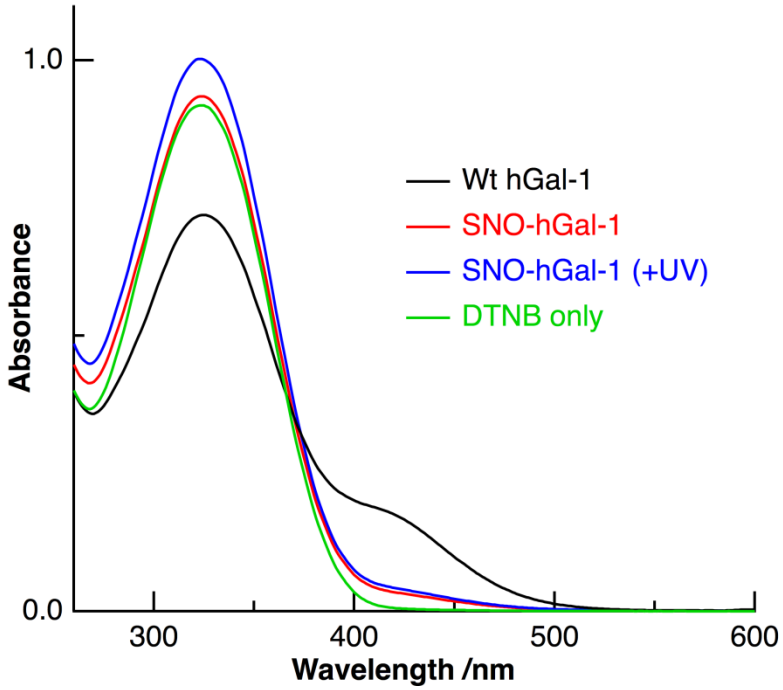

Fig. S5. Absorption spectra of DTNB with WT hGal-1 (black), SNO-hGal-1 before (red) and after (blue) UV photoirradiation, together with that of DTNB only in buffered solution.

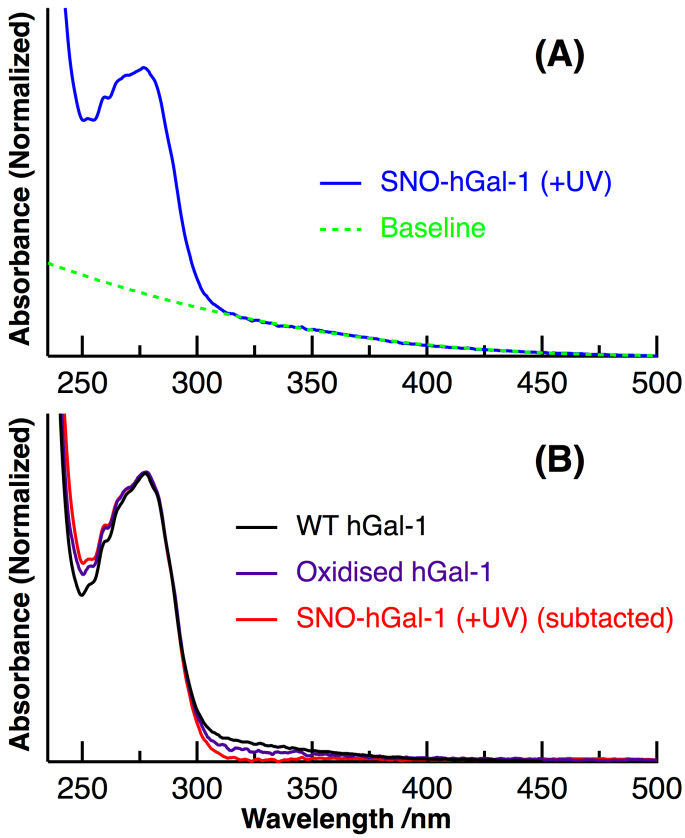

Fig. S6. (A) Absorption spectrum of photoirradiated SNO-hGal-1 (blue) and the baseline (green). (B) Absorption spectra of WT hGal-1 (black), oxidised WT hGal-1 (purple) and the difference spectrum obtained by the subtraction of the baseline from the spectrum of photoirradiated SNO-hGal-1 (red). The absorption spectra of WT hGal-1 and SNO-hGal-1 are the same as those of Fig. 1.

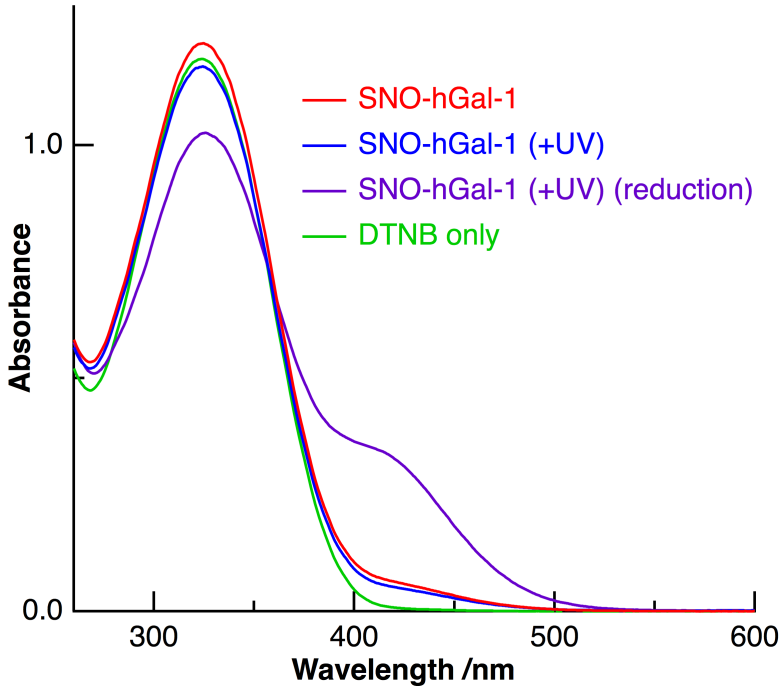

Fig. S7. Absorption spectra of DTNB with SNO-hGal-1 before (red) and after (blue) UV photoirradiation, and those of DTNB with photoirradiated SNO-hGal-1 after the reduction by DTT (purple) and of DTNB only (green).

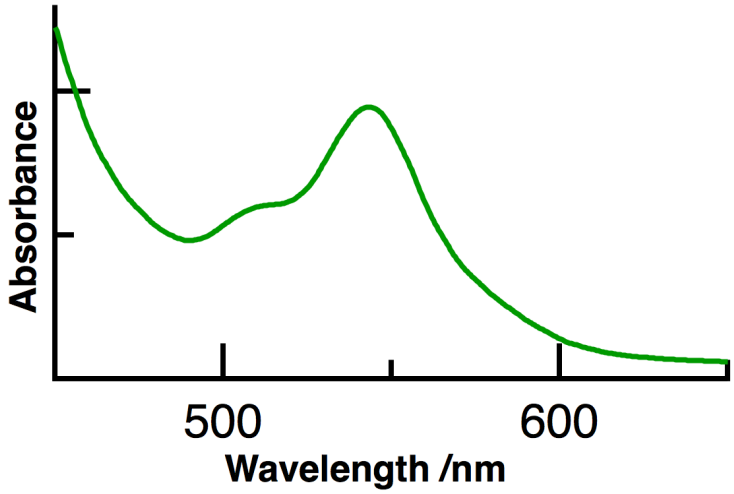

Fig. S8. Absorption spectrum of SNO-Cys in the visible region, which is shown as the example of the visible absorption of SNO.

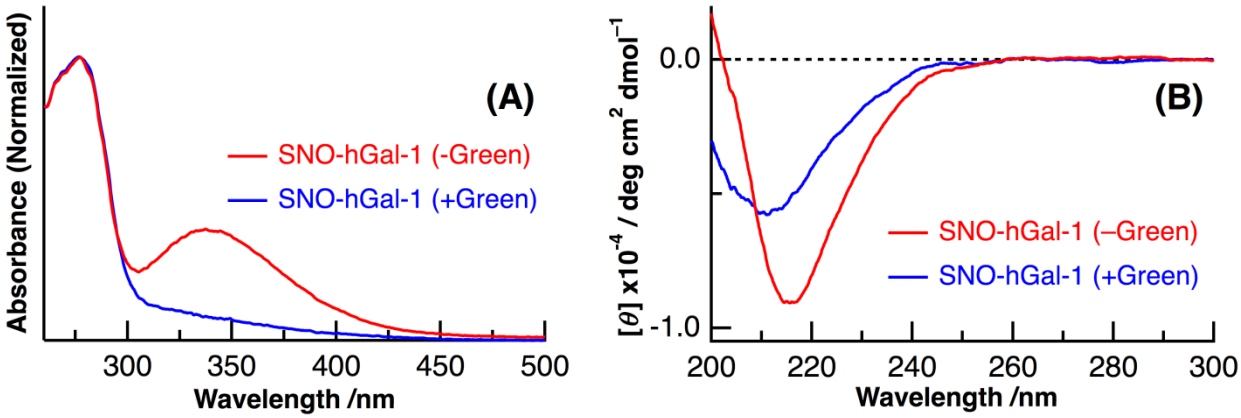

Fig. S9. (A) Absorption spectra of SNO-hGal-1 prepared by SNO-MT without (red) and with (blue) visible photoirradiation. (B) CD spectra of SNO-hGal-1 prepared by SNO-MT without (red) and with (blue) visible photoirradiation.

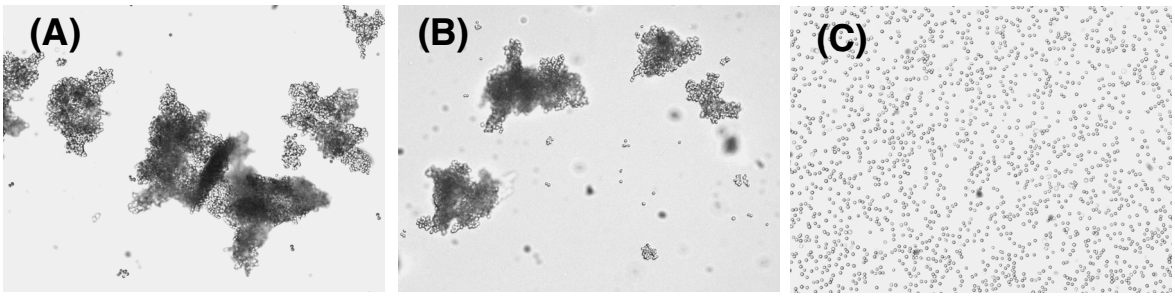

Fig. S10. (A, B) Aggregates of sheep erythrocytes formed by WT hGal-1 in the presence of SNO-Cys before photoirradiation (A) and after photoirradiation with 355 nm light for 60 min (B). (C) Sheep erythrocytes after the incubation with oxidised WT hGal-1.

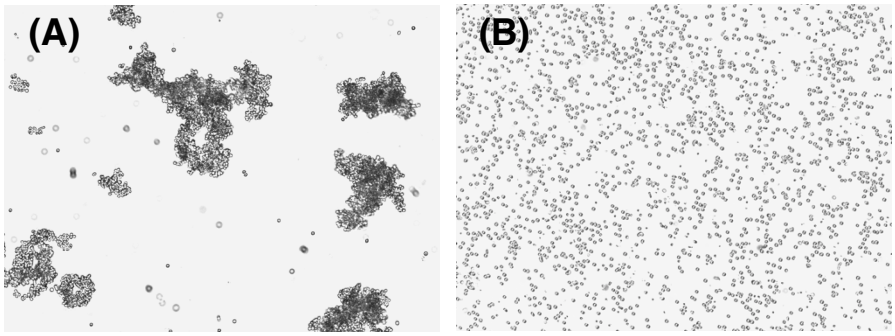

Fig. S11. Effect of photoirradiation with 532 nm light on aggregates of sheep erythrocytes formed by SNO-hGal-1. (A): without photoirradiation for 90 min, (B): with photoirradiation for 90 min.

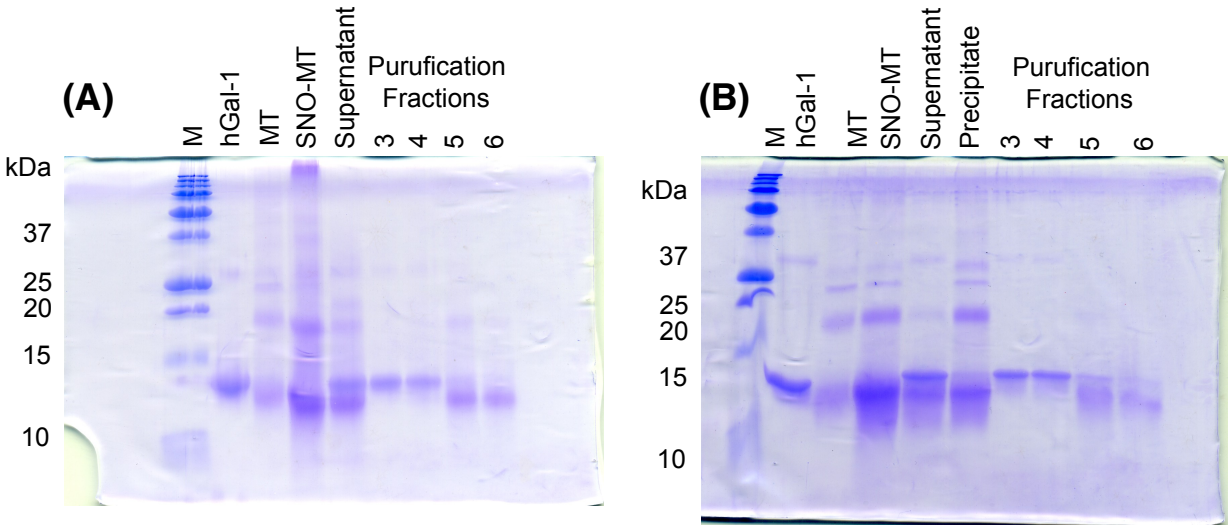

Fig. S12. Non-reduced (A) and reduced (B) SDS-PAGE during the reaction of WT hGal-1 and SNO-MT. The complex between hGal-1 and MT was not observed in supernatant in (A), indicating that the amount of the complex between hGal-1 and MT with the disulfide bond was negligibly small. The lane of precipitate dissolved by DTT in (B) has a strong band due to MT, indicating that the precipitate after the reaction is mainly due to the aggregates of MT formed with intra- and inter-disulfide bonds.

3. References

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4. Author Contributions

T.N. and H.H. conceived and conducted the research. T.K., I.S. and K.K. carried out the experiments and analyzed the data. T.N. and H.H. checked the analyzed data. T.N. wrote the manuscript. T.K, K.K, and H.H checked the manuscript.