Supporting Online Material

Chemical Characterization of the Smallest *S*-nitrosothiol, HSNO; Cellular Cross-talk of H_2S and *S*-nitrosothiols

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Supporting text

In order to calculate the standard Gibbs energy of the reaction

$$GSNO + H_2S \rightarrow GSSH + HNO \quad (1)$$

we split the reaction in 6 parts:

$GSNO$	$\rightarrow$	$GS^{\bullet} + NO^{\bullet}$	+110 kJ/mol[1]	(2)
H_2S	$\rightarrow$	$S^{\bullet-} + 2H^+ + e^-$	–0.92 V at pH 7[2]	(3)
$S^{\bullet-} + H^+$	$\rightarrow$	$HS^{\bullet}$	?	(4)
$GS^{\bullet} + HS^{\bullet}$	$\rightarrow$	$GSSH$	?(+208 kJ/mol)	(5)
$GSSH$	$\rightarrow$	$GSS^- + H^+$	?	(6)
$NO^{\bullet} + H^+ + e^-$	$\rightarrow$	HNO	?(–0.51 V at pH 7)	(7)

While the energetics of the the first two reactions are known, those of the last four need to be estimated.

In order to estimate standard Gibbs energy of the S–S bond in GSSH, we determine the difference with that in GSSG, 226 kJ/mol. This value follows directly from the electrode potentials of the couples $GS^{\bullet}$/GSH (+0.94 V)and GSSG/2GSH, –0.23 V.[3] We approximate GSSG with CH_3SSCH_3, and GSSH with CH_3SSH, and calculate, with group additivities of Benson,[4] the enthalpies of formation in the gas phase The values are –12.7 kJ mol^{-1} for CH_3SSCH_3, and –4.7 kJ mol^{-1} for CH_3SSH. We then react these two compounds with H_2:

$CH_3SSCH_3 + H_2$	$\rightarrow$	$2\ CH_3SH$	$\Delta_{rxn}H° = –20.3$ kJ mol^{-1} (8)
$CH_3SSH + H_2$	$\rightarrow$	$CH_3SH + H_2S$	$\Delta_{rxn}H° = –38.2$ kJ mol^{-1} (9)

to produce CH_3SH (–22.8 kJ mol^{-1})and H_2S (–20.1 kJ mol^{-1}) and oxidize them both by one electron. The enthalpy difference, 18 kJ/mol ought to be the difference in bond energy, that of CH_3SSH being smaller, 226 – 18 = 208 kJ/mol. As these reactions are carried out in parallel, all errors (mixing enthalpies with Gibbs energies, and ignoring Gibbs energies of solution) should cancel. The energetics of the oxidation of GSH and H_2S by one electron are within the error identical, thus the difference in bond energy follows from the difference in the enthalpies of reduction of CH_3SSCH_3 and CH_3SSH with H_2.

The pK_a's of $HS^{\bullet}$ and that of CH_3SSH, reactions 4 and 6 are not known. They are very likely less than 7, and we assume that they cancel. The estimated error of this assumption is 11 kJ mol^{-1}, which would reflect a difference of 2 pH units.

The standard electrode potential of the $NO^{\bullet}/NO^-$ couple is –0.8 V; the pK_a of HNO is > 11.5,[5] and 12 was used. Given that $\Delta_f G°(NO^{\bullet})$ is + 102 kJ kJ mol^{-1},[1] then $\Delta_f G°(NO^-)$ is +179 kJ mol, both in water. That of HNO is then +111 kJ kJ mol^{-1} at pH 0, +151 kJ kJ mol^{-1} at pH 7, and thus $E°'(NO^{\bullet}, H^+/HNO)$ is –0.5 V. The error is estimated at 0.1 V. Addition of the energetics of Reaction 2-7 yields $\Delta_{rxn1}G° = +40$ kJ mol^{-1}.

Reference List

1. Koppenol, W. H. Nitrosation, thiols, and hemoglobin: Energetics and kinetics. *Inorg. Chem.* **2012,** *51*, 5637-5641.

2. Das, T. N.; Huie, R. E.; Neta, P.; Padmaja, S. Reduction potential of the sulfhydryl radical: Pulse radiolysis and laser flash photolysis studies of the formation and reactions of .SH and HSSH.$^-$ in aqueous solutions. *J. Phys. Chem. A* **1999,** *103*, 5221-5226.

3. Koppenol, W. H. A thermodynamic appraisal of the radical sink theory. *Free Radical Biol. Med.* **1993,** *14*, 91-94.

4. Benson, S. W. *Thermochemical Kinetics;* 2 ed.; John Wiley & Sons: New York, 1976.

5. Farmer, P. J.; Kumar, M. R.; Almaraz, E. The coordination chemistry of HNO: From Warren Roper to hemoglobin. *Comments on Inorganic Chemistry* **2010,** *31*, 130-143.

Supporting table

Table S1. Computed absolute energies (hartree) and zero-point vibrational energies (ZPVE, kcal mol^{-1})

	E, Hartree	ZPE, kcal/mol	G, Hartree
Complex 1	-967.53938	37.86	-967.51857
Complex 2	-966.98458	32.27	-966.96814
TS1	-967.49976	36.84	-967.47333
TS2	-967.46329	37.60	-967.43789
TS3	-966.97533	32.26	-966.95714
TS4	-966.91952	31.28	-966.90394
Final 1	-967.52830	38.25	-967.50753
Final 2	-967.52075	40.08	-967.49146
Final 3	-967.00568	33.04	-966.98928
Final 4	-966.98656	34.79	-966.96486

Gaussian Archives

Complex 1

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416534,1.7972636486,0.3143833228\C,-1.5145674399,-0.9115157999,0.76681
72672\H,-1.9916376226,-0.1301700743,1.3636372277\H,-2.1830304831,-1.76
41272645,0.6804061455\H,-0.5757237199,-1.2068972767,1.2283050494\S,2.6
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t=0.0603352\Thermal=0.0703416\Dipole=-0.0172494,-0.7827291,0.1114791\D

TS1

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TS2

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,0.0968732304\C,0.1056627071,-0.855467161,-0.0220222049\H,1.1663632392
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\H,-0.3220442084,-1.4146163107,-0.8481077552\S,-2.7757880476,0.9276485
011,0.474207456\H,-2.8085564914,-0.3532230839,0.8898601682\H,-0.557148
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MeSNO

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anti

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9076\H,2.20034,0.02061,-0.89076\H,1.10697,1.10881,-0.00009\\Version=EM
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H_2S

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Anionic

Complex 2

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TS3

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TS4

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Final 3

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Final 4

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19411326\H,0.5996383118,2.5482347779,-0.170701753\S,-1.5681606656,-0.8
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HS^-

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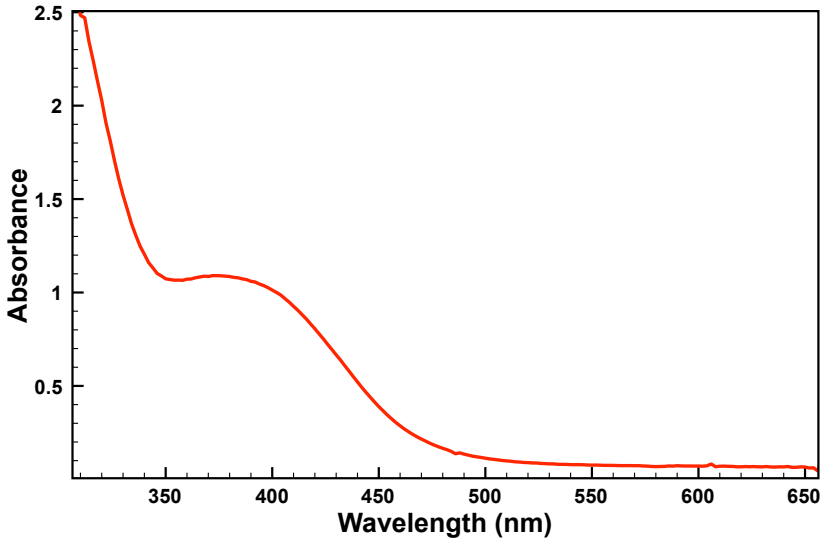
Supporting figures

A



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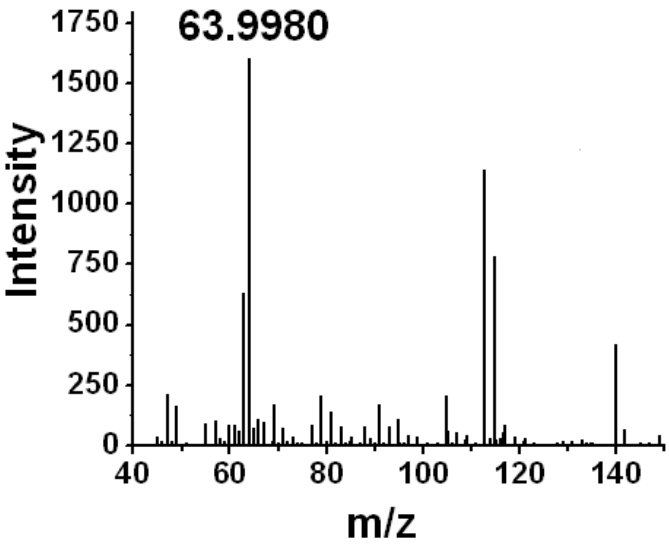
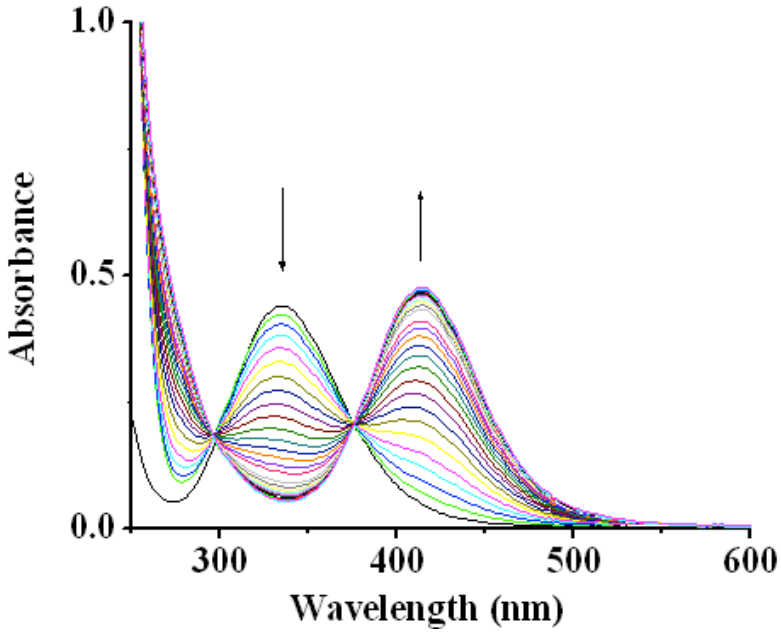
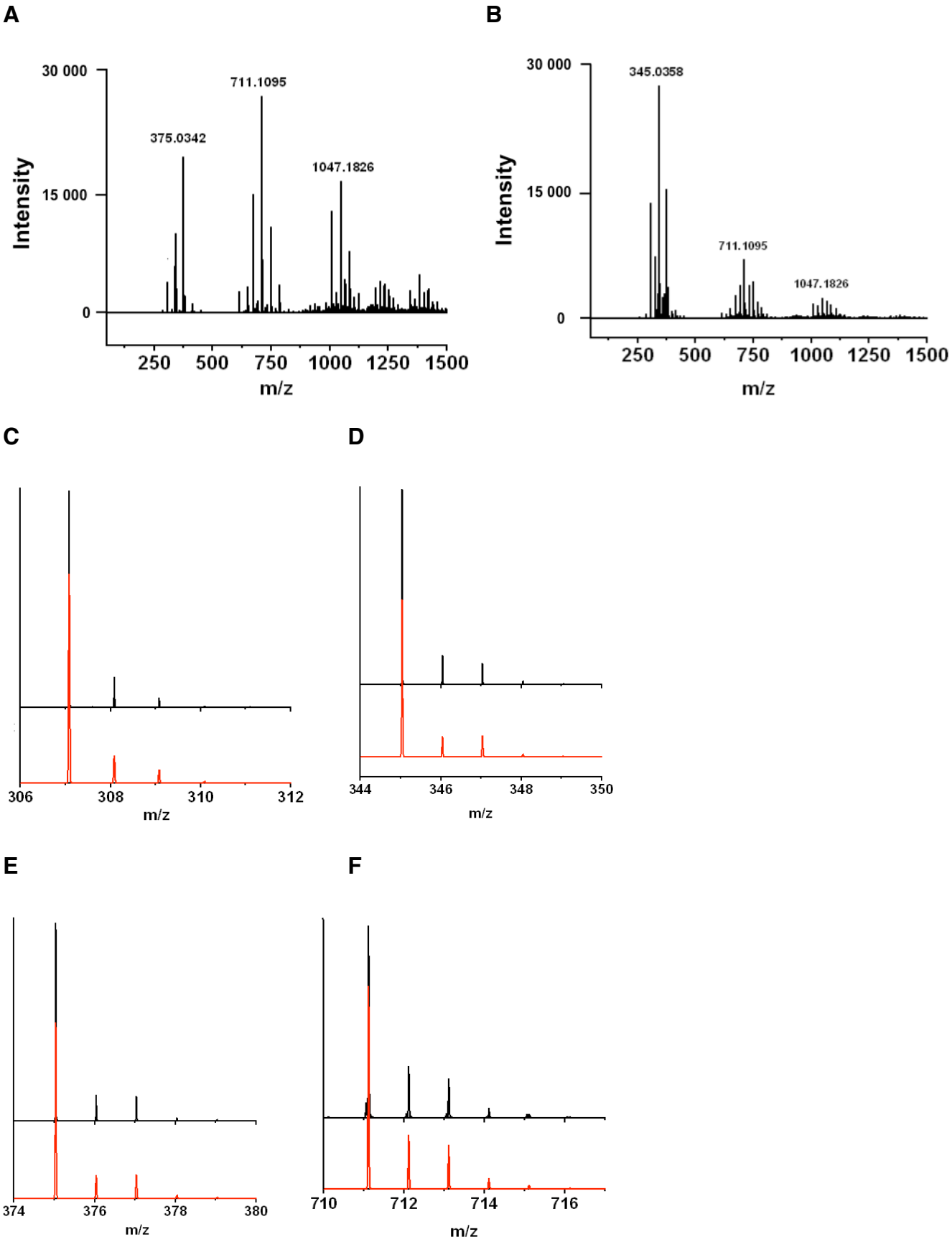


Figure S1. Generation of HSNO by acidification of solution containing nitrite and sulfide. A) UV-Vis spectrum of neutralized solution of HSNO. HSNO was prepared by acidification of 100 mM nitrite with 100 mM HCl, followed by addition of sodium sulfide (final concentration 10 mM). This led to a colour change characteristic of S-nitrosothiol formation. The solution was rapidly neutralized using 300 mM phosphate buffer pH 7.4. The broad peak at 370 nm represents a mixture of unreacted nitrite (350 nm), HSNO (335 nm, determined by pulse radiolysis in this study) and $HSS^{\cdot 2-}$ (380 nm). **B) Mass spectrum of HSNO prepared by acidification of nitrite in the presence of sulphide.** The peak at m/z 64 was assigned to $[HSNO + H^+]^+$.



<u>Supplementary Figure S2.</u> Time resolved UV-Vis spectra (collected every 4s) at 37 °C of buffered *S*-nitroso glutathione (0.5 mM) upon addition of sodium sulfide (0.5 mM). Decrease of the characteristic *S*-nitroso peak at 334 nm parallels the appearance of the new species at 412 nm which does not correspond to HSNO but, instead, to the mixed polysulfides as explained in the main text.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500
750
1000
1250
1500
m/z
B
30 000
15 000
0
Intensity
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711.1095
1047.1826
250
500
750
1000
1250
1500
m/z
C
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308
310
312
m/z
D
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m/z
E
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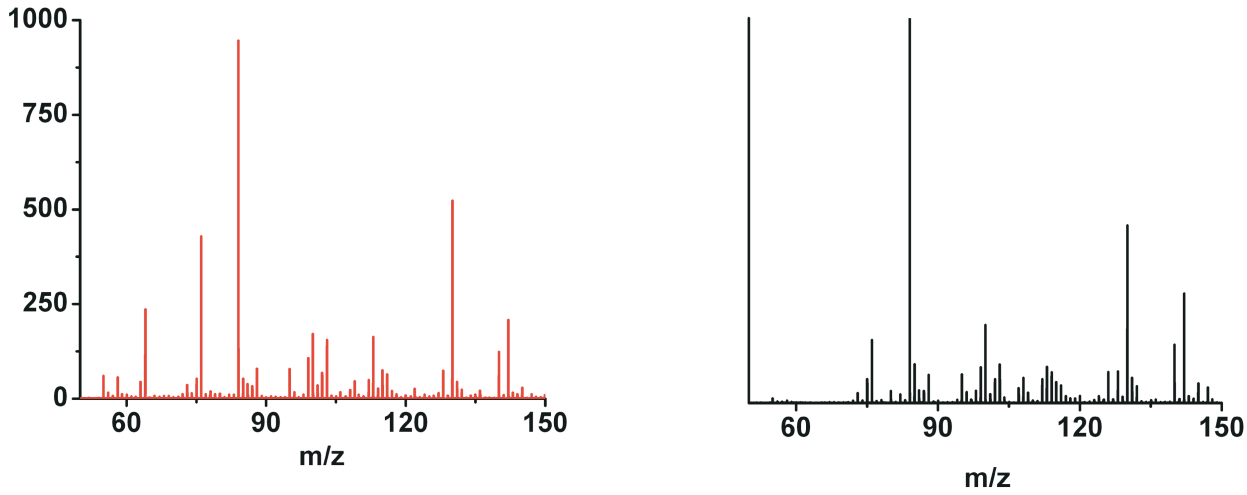
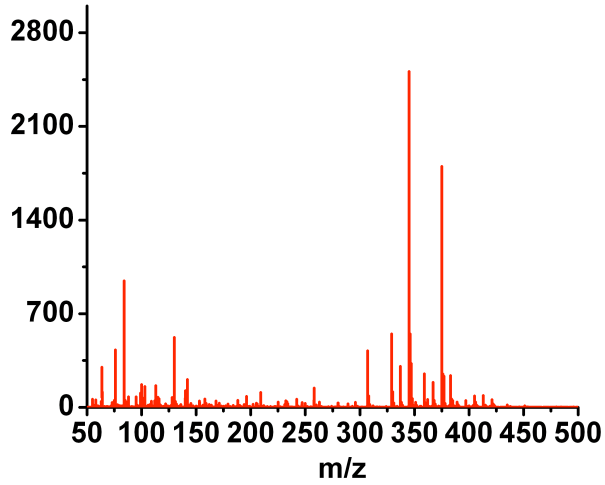
376
378
380
m/z
F
710
712
714
716
m/z

G

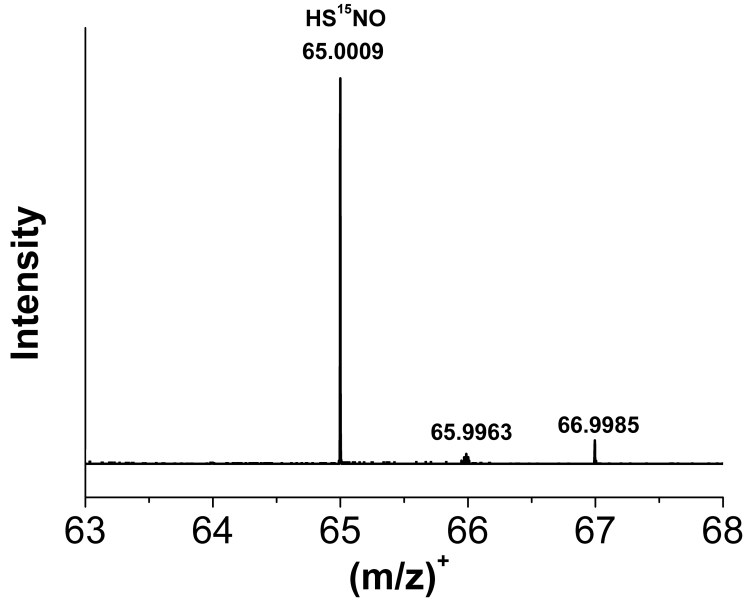


H

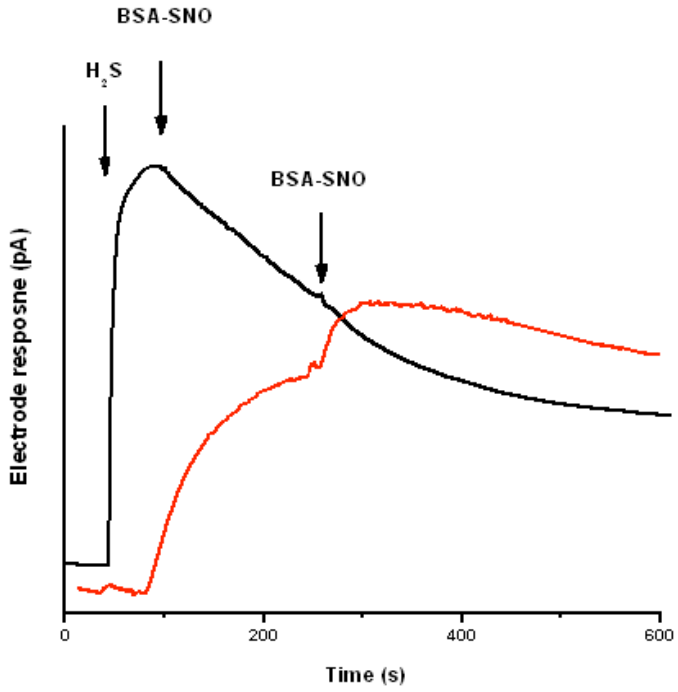


Supplementary Figure S3. ESI-TOF mass spectrometry of the reaction mixture containing GSNO and H_2S. a) Mass spectrum of GSNO. *S*-Nitrosoglutathione gives three particularly intense peaks at *m/z* 375.0342, 711.1095 and 1047.1826, which correspond to $[GSNO]K^+$, $[GSNO]_2K^+$, and $[GSNO]_3K^+$, respectively. **b) Mass spectrum of GSNO mixed with H_2S.** The major peak at *m/z* 345.0358 is assigned to decomposed *S*-nitroso glutathione $[GS^{\cdot} + K]^+$ or $[GSSG + 2K]^+$. **c-f) Comparison of the original (black) spectrum with the simulated one (red) for peaks at c) *m/z* 307 $[GS^{\cdot} + H^+]^+$, d) *m/z* 345 $[GS^{\cdot} + K^+]^+$, e) *m/z* 375 $[GSNO + K]^+$ and f) *m/z* 711 $[GSNO]_2K^+$. g) Low mass range spectral analysis of the reaction mixture containing GSNO an H_2S.** The spectra are collected in a mode that focuses on low mass range only in a solution containing 150 mM potassium phosphate buffer. The arrow indicates appearance of a *m/z* 64 signal, assigned to $[HSNO + H^+]^+$ in the spectrum of the

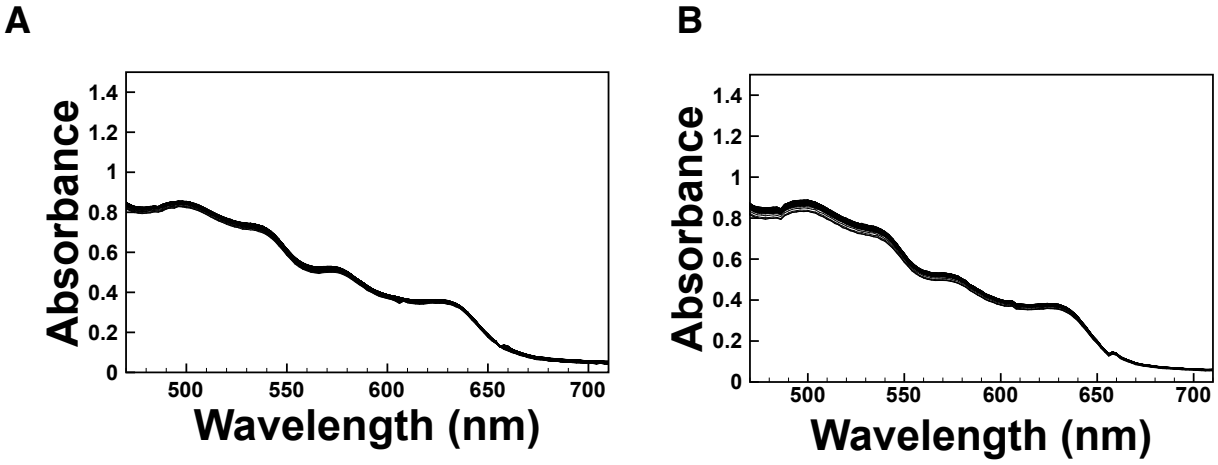
mixture (red) that is absent in the control spectrum of GSNO (black). **h) The whole low mass range spectrum presented for comparison.**



Supplementary Figure S4. Mass spectrum of $HS^{15}NO$ prepared by mixing $GS^{15}NO$ and H_2S. 0.3 equiv portion of H_2S was mixed with buffered 1 mM $GS^{15}NO$ (pH 7.4), protected from light and injected directly into the maXis (Bruker Daltonics), high-resolution mass spectrometer.



Supplementary Figure S5. Amperometric recordings from NO (red) and H_2S electrode (black) measurements during the reaction of hydrogen sulfide with *S*-nitroso albumin. A 50 μM solution of sodium sulfide was injected into 50 mM potassium phosphate buffer, pH 7.4. After some time, *S*-nitrosoalbumin (prepared by transnitrosation of albumin with *S*-nitrosocysteine and then dialyzed for 24 h in dark) was injected as indicated by the arrows. Decay of H_2S from the solution (black line) parallels the rise of the signal from an NO electrode (red line).



Supplementary Figure S6. Spectral changes of methemoglobin upon addition A) GSNO and B) just H_2S. 100 μM GSNO or 200 μM sodium sulphide were added to 50 μM metHb in 50 mM potassium phosphate buffer pH 7.4 and the reaction was followed every 10 s for a total of 5 min.

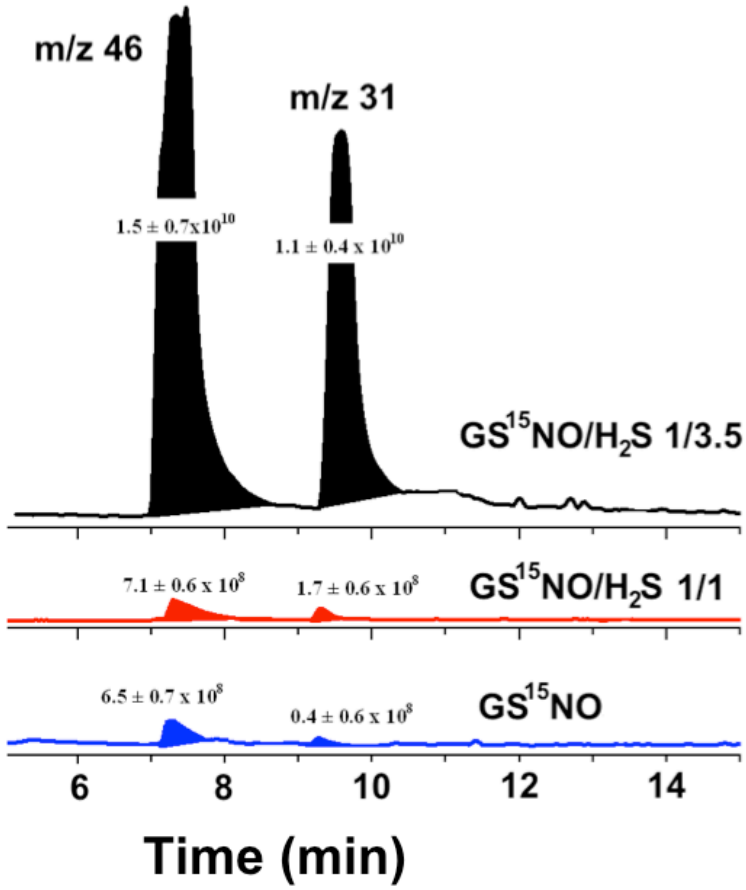
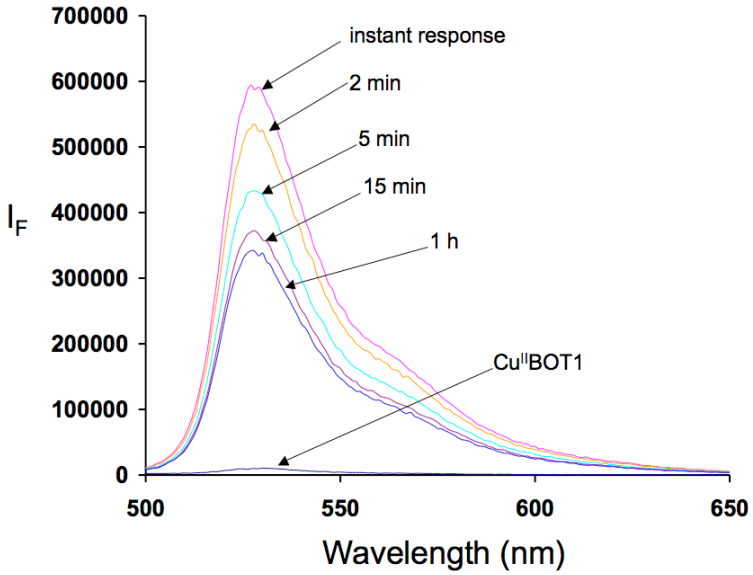
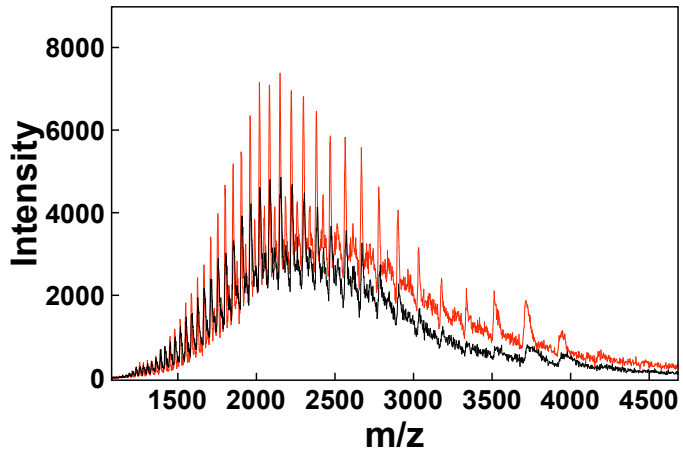


Figure S7. N_2O and $NO^{\cdot}$ formation from the reaction of $GS^{15}NO$ with Na_2S. A 5 mM $GS^{15}NO$ solution in 50 mM potassium phosphate buffer, pH 7.4, (blue) was degassed with argon and mixed with Na_2S to give a ratio of 1:1 (red) or 1:3.5 (black). Fifteen min after sulfide addition, the headspace gas was analyzed for ions m/z 46 ($^{15}N_2O$) and 31 (^{15}NO) by GC-MS. The values given in the figure represent the calculated areas under the corresponding curves.



Supplementary Figure S8. ***In vitro*** **reaction of CuBOT1 with H_2S.** Intracellular formation of HNO was visualized using CuBOT1, a fluorescent sensor for nitroxyl.[16] The ability of H_2S to reduce CuBOT1 directly, in vitro, was also tested. The figure presents the fluorescence response of 3 μM CuBOT1 to 300 μM H_2S (λ_{ex} = 450 nm). The spectra were acquired in 50 mM PIPES buffer, 100 mM KCl, pH 7.0, 37 °C. However, as for other reducing agents (glutathione and cysteine),[16] this response does not occur inside the cells, making this probe suitable for HNO detection under the experimental conditions used in this study

A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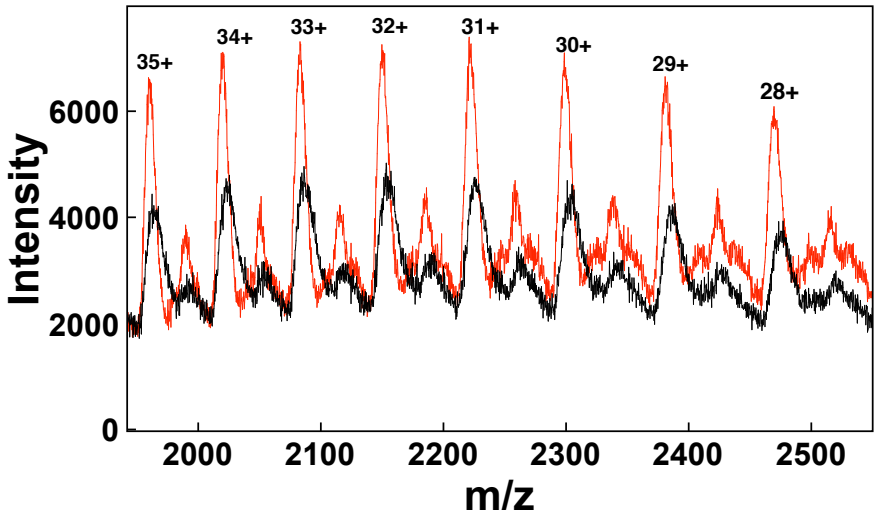
2000
2500
3000
3500
4000
4500
m/z

B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34+
33+
32+
31+
30+
29+
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0
2000
2100
2200
2300
2400
2500
m/z

C

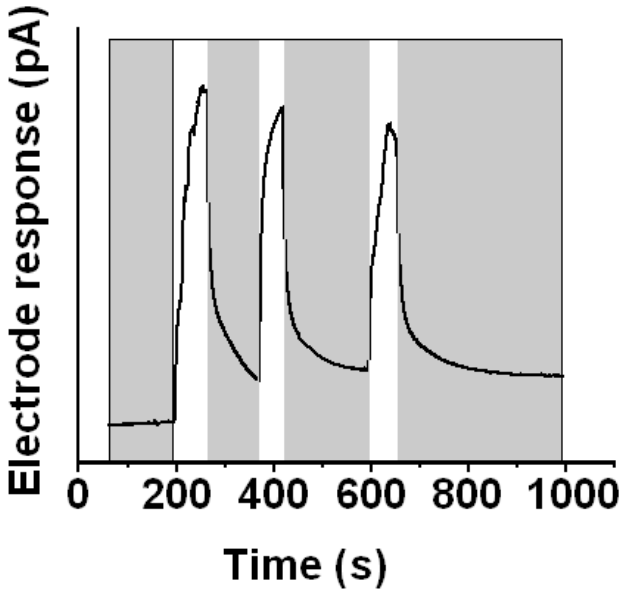
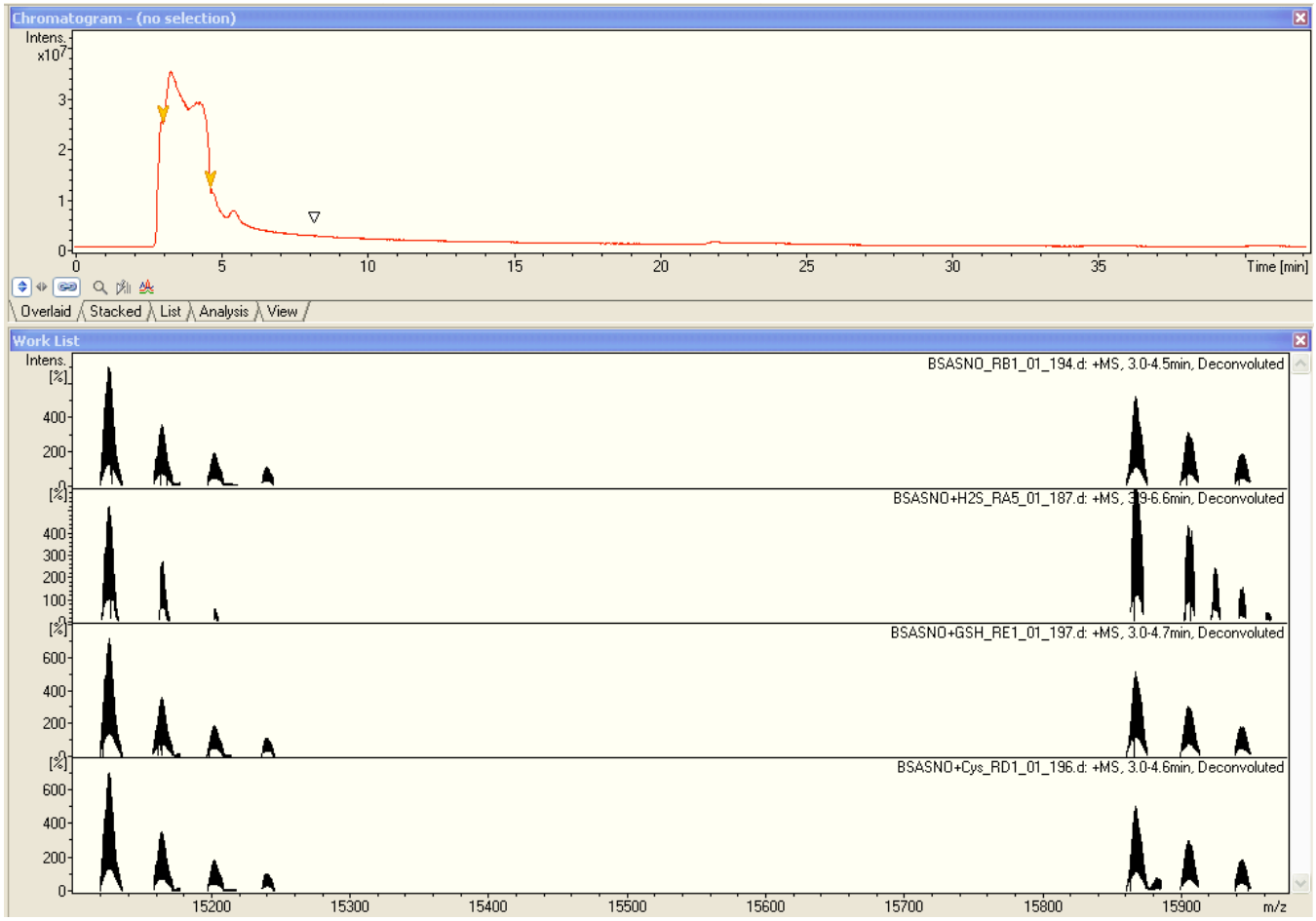

Electrode response (pA)
0
200
400
600
800
1000
Time (s)

Figure S9. A) ESI-TOF mass spectrum of *S*-nitroso-albumin obtained by reaction of albumin with HSNO formed from *S*-nitrosoglutathione and sulfide. A 1 mM solution of GSNO was incubated with 1 mM Na_2S in 300 mM potassium phosphate buffer, pH 7.4, for 5 min, to assure that all GSNO reacted to form HSNO. Next, 200 μM bovine serum albumin (BSA) was added to the solution and allowed to incubate for 15 min. The solution was dialyzed against water for 24 h and prepared for mass spectrometry in an acetonitrile/methanol mixture (1:1, v/v) containing 0.1 % formic acid. The red trace represents the mass spectrum of native BSA, the black trace is the spectrum of HSNO-treated protein. **B) Enlarged central part of the spectrum.** For example, the 31^+ peak was shifted upwards by m/z 3.77 which corresponds well to the value calculated for the addition of 4 nitroso equivalents ($[BSA - 4H + 4NO]^{31+}$ = [4 x 29]/31 = 3.74). **C) Light activated NO· release from poly-nitrosated bovine serum albumin.** Polynitrosated albumin releases NO· on exposure to light, as detected by an NO electrode. Subsequent irradiation of the same sample led to gradual reduction in NO quantities released due to decreasing amounts of *S*-nitrosated material remaining.

A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x10^7
Time [min]
Overlaid
Stacked
List
Analysis
View
Work List
Intens.
[%]
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BSASNO+H2S_RA5_01_187.d: +MS, 3.9-6.6min, Deconvoluted
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m/z

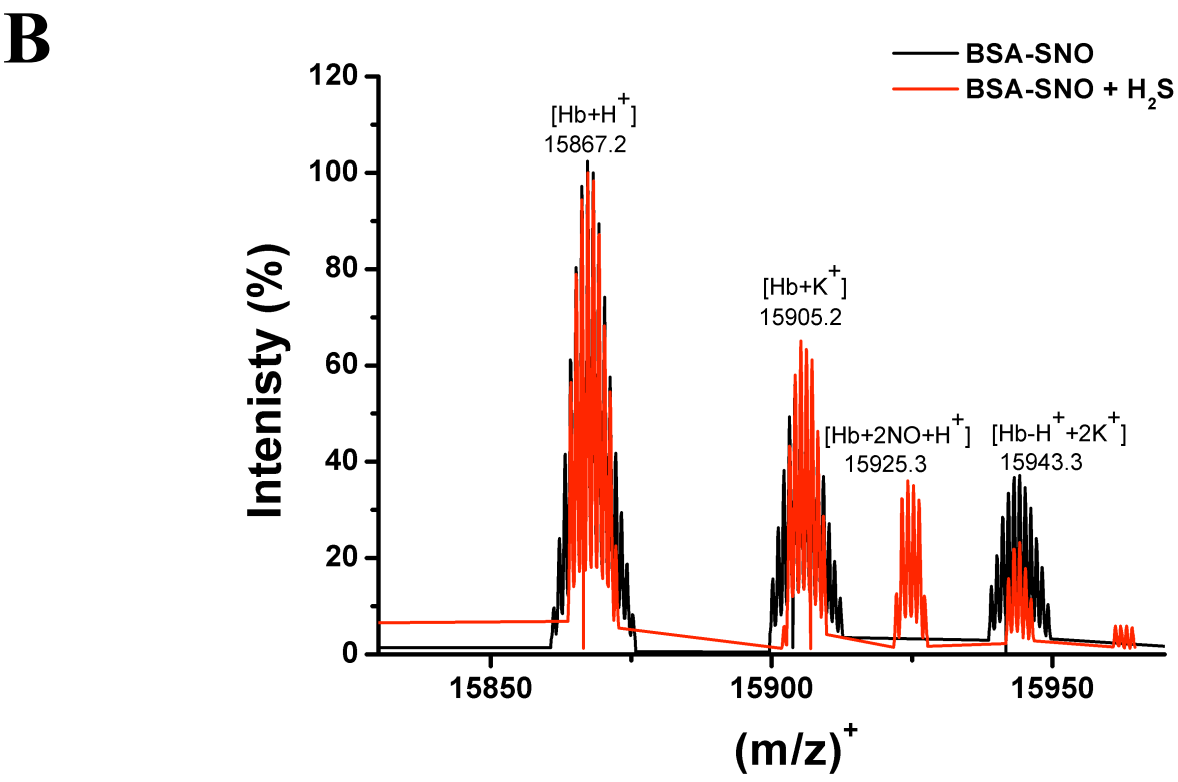

Figure S10. LC-ESI-TOF MS analysis of hemoglobin from red blood cells (RBC) treated with BSA-SNO, BSA-SNO + H_2S, BSA-SNO + GSH and BSA-SNO + Cys. A) Original data obtained from the experiments, analyzed by Data Analysis software (Bruker Daltonics). The spectra depict MS chromatogram and corresponding spectra of hemoglobin (Hb) alpha and beta subunits. Only when the combination of BSA-SNO and H_2S was used, an additional peak was observed assigned to $[\beta Hb+2NO+H]^+$. No changes in αHb were observed in any of the samples. B) Enlarged spectrum of βHb after the RBC were treated with BSA-SNO only (black) or BSA-SNO + H_2S (red). 20 μM washed RBC were diluted 100x with PBS and exposed to 100 μM poly BSA-SNO (prepared by acidification of BSA/nitrite mixture and then further purified on micro Bio-Spin column) without or with addition of 100 μM H_2S, 100 μM GSH or 100 μM Cys. 2 min after the addition of BSA-SNO the samples were centrifuged for 3 min at 3000x*g* and then washed with PBS 3 times prior the hemolysis with nanopure water supplemented with 100 μM neocuproine. Samples (20 μL) were separated on LC and subsequently analyzed by ESI-TOF-MS. LC separation was done using following protocol: column was equilibrated with a 50:50 mixture of buffer A (80:20, water:acetonitrile; 0.1% trifluoroacetic acid) and buffer B (40:60, water:acetonitrile; 0.1% triflu- oroacetic acid) at a flow rate of 1 ml/min. 20 μL of each sample was loaded and eluted with a 2-min hold at 50% B, followed by a linear gradient to 66% B over 40 min.