Electronic Supplementary Information (ESI)

For

The Rational Design of Peptide-based Hydrogel Responsive to H_2S

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1. Material and Methods

1.1. Reagents and instruments

All chemicals and solvents were purchased from commercial suppliers and used without further purification. Reaction progress was monitored by TLC on pre-coated silica plates (Merck 60 F254, 250 μm thickness) and spots were visualized by UV light. Merck silica gel 60 (70-200 mesh) was used for column chromatography purification. ^{1}H NMR spectra were recorded on a Bruker model DPX-300 MHz or DPX-400 MHz NMR spectrometer. Chemical shifts are reported in parts per million relative to internal standard tetramethylsilane ($Si(CH_3)_4$ = 0.00 ppm). ^{1}H NMR coupling constants (J) are reported in Hertz (Hz), and multiplicity is indicated as following: s (singlet), d (doublet). Mass spectra (MS) were obtained on a PE Sciex API 150EX LC/MS system. HPLC purification was carried out on a 1525 Waters HPLC system equipped with a 2489 UV/Visible Detector. The pH of solutions was monitored using a Hanna HI 2211 pH meter equipped with a Toledo U402-M3-S7/200 electrode.

1.2. Preparation of H_2S solutions

H_2S solutions were generated by dissolving $Na_2S.xH_2O$ in either deoxygenated PBS or pure water. The pH was then adjusted to 7.4 with concentrated HCl.[1] Under pH = 7.4 and 37 ℃, around 20% of sulfide is present as H_2S. In this study, we quantified the concentration of total sulfide species (S^{2-} + HS^- + H_2S) in solution following the reported procedure.[1] For each solution, the concentration of total sulfide species in solution was determined by UV measurement of a solution sample diluted in deoxygenated carbonate buffer at pH = 9.6 (the sulfide is present entirely as HS^- when pH is 9.6, and only HS^- species has UV absorbance at 230 nm). The absorbance was measured at 230 nm, and a molar absorptivity value of 7200 mol^{-1} L cm^{-1} was used for the calculation.[2]

1.3. Hydrogel preparation and H_2S assay in vial

Hydrogelation experiments were carried out in glass vials. The appropriate amount of powder peptide **1**-**5** was first dissolved in a small quantity of DMSO and immediately diluted with MilliQ water so as to obtain a final concentration of DMSO equal to 5%. After a few minutes, hydrogel formation was tested by the vial inversion method.

To test the effect of H_2S on a bulk quantity of gel (as presented in Figure 1), two samples of 200 μL of a hydrogel containing 0.5% wt of **1** in pure water + 5% DMSO were prepared in two separate vials and allowed to stand at room temperature for a few minutes. 40 μL of a fresh solution of H_2S (1.22 M) in PBS (1 M; pH = 7.4)

was then deposited on top of one of the hydrogel and the mixture was incubated for two hours at 37 °C. 40 µL of PBS (1 M; pH = 7.4) was added to the control sample instead. Again, the vial inversion method was used to test the gel degradation.

1.4. TEM observations

Two samples of 100 µL of a hydrogel containing 0.2% wt of **1** in pure water + 5% DMSO were prepared in two separate vials and allowed to stand at room temperature for a few minutes. 25 µL of a fresh solution of H_2S (0.82 M) in MilliQ water (pH adjusted to 7.4) was then added in one of the vial. 25 µL of MilliQ water (pH adjusted to 7.4) was added in the control vial. Both vials were then incubated overnight at 37 °C. For each samples, a 10 µL drops was then deposited onto a TEM carbon support and allowed to dry overnight. Transmission electronic microscopy (TEM) was conducted on a Philips Technai 12 Transmission Electron Microscope under an accelerating voltage of 120 KV.

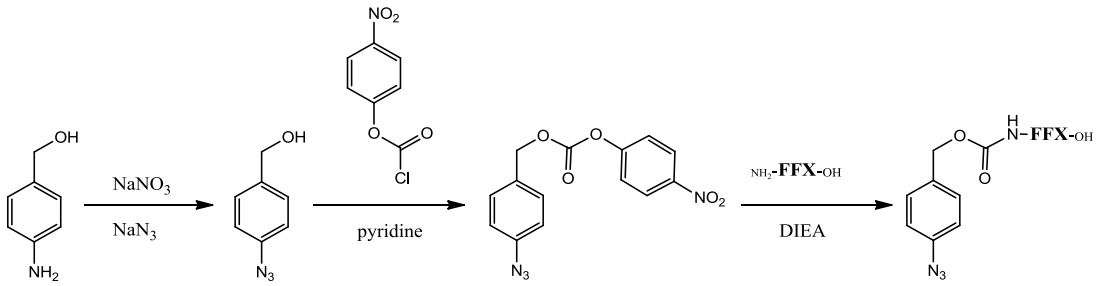
1.5. HPLC monitoring of azidobenzyl-carbamate removal by H_2S

Degradation of a solution of peptide **1** in the presence of H_2S as a function of time, or as a function of H_2S equivalents, was monitored by High Pressure Liquid Chromatography (HPLC) at 254 nm. Fresh solutions of H_2S (152, 110, 69, 48, 28, 15 and 7 mM) or GSH (165 and 83 mM) were prepared in PBS (1 M; pH = 7.4) and further adjusting the pH to 7.4 using concentrated HCl or concentrated NaOH. 22 µL of the analyte solutions was then added to 200 µL of a solution of peptide **1** (0.36 mM) in water + 5% DMSO. For the kinetic experiment, a sample was immediately taken for analysis and the solution was put to stir at 37 °C for 2 hours. At regular intervals of time, 20 µL samples were taken out of the mixture and analysed by analytical HPLC at 254 nm. For monitoring of the effect of analyte concentration (H_2S or GSH), the samples were shaken mildly at either 25 °C or 37 °C overnight instead, and injected in the HPLC for analysis. For both experiments, the percentage of **1** remaining was calculated using the area of integration value of the peak corresponding to peptide **1** at 254 nm. When appropriate, HPLC peaks were isolated, freeze dried, re-dissolved in methanol and analysed via MS for characterisation.

1.6. Characterization of gel degradation by various analytes

On a glass slide, 0.8 µL drops of a stock solution of **1** at 0.1 mg/µL in DMSO were spotted and immediately diluted with 19.2 µL of MilliQ water to form small, domed-shaped hydrogels (0.4% wt). H_2S solution of 0.18 M, 0.43 M and 1.22 M, and a solution of GSH at 2M, all in PBS (1 M, pH = 7.4) were freshly prepared prior the experiment and adjusted to pH = 7.4 using concentrated HCl or concentrated NaOH. Drops of 5 µL of the analyte solutions were then deposited onto the gels using a pipette tip, the glass slides sealed off in individual dishes and the samples incubated at 25 °C or 37 °C. After the indicated periods of time, the slides were washed carefully by immersion in a beaker of water. Gels spots degraded by high levels of H_2S were eventually washed away during this step. Immediately after taking a picture of the resulting slides, and before the hydrogels could dry, a fresh drop (5 µL) of the analyte solutions was added to each gels, which were then sealed off and further incubated. For the control sample, a 5 µL drop of PBS (1 M, pH = 7.4) was added instead of the analytes.

2. Synthesis of azidobenzyl-carbamate modified peptides



2.1. Synthesis of (4-azidophenyl)methanol (5)

A solution of 4-aminobenzyl alcohol **4** (400 mg, 3.25 mmol) in 2.5 mL HCl (5M) was cooled to 4 ℃ and a solution of sodium nitrite (269 mg, 3.9 mmol) in 10 mL water was added dropwise over 30 minutes. The mixture was further stirred for 30 minutes at 4 ℃. A solution of NaN_3 (253 mg, 3.9 mmol) in 5 mL water was then added dropwise over 30 minutes while keeping the temperature at 4 ℃, following which the mixture was allowed to warm at room temperature and was further stirred for 2h. The reaction was then quenched with brine, extracted with ethyl acetate and washed with a saturated solution of $NaHCO_3$, brine and water, dried over $MgSO_4$ and the solvent evaporated. Crude product was purified by flash chromatography (dichloromethane:methanol 20:1) to give the desired product **5** as yellow oil (359 mg, 74% yield). ^{1}H NMR ($CDCl_3$): δ= 1.84 (s, 1H; OH), 4.68 (s, 2H; CH_2), 7.04 (d, J = 9.0 Hz, 2H, aromatic), 7.36 (d, J = 9.0 Hz, 2H; aromatic). MS: m/z: 150.0 $[M+H]^+$. Spectroscopic data for the title compound were consistent with the literature.[3]

2.2. Synthesis of 4-azidobenzyl 4-nitrophenyl carbonate (6)

4-nitrophenyl chloroformate (243 mg, 1.2 mmol) and pyridine (190 mg, 2.4 mmol) were dissolved in 6 mL dry tetrahydrofuran (THF) and cooled to 4 ℃. A solution of 4-azidobenzyl alcohol **5** (180 mg, 1.2 mmol) in 9.5 mL dry THF was then added dropwise over 30 minutes while keeping the temperature at 4 ℃, following which the mixture was allowed to warm at room temperature and was further stirred overnight. The solvent was then evaporated and 30 mL of ethyl acetate was added. The mixture was then washed with 10% $NaHCO_3$ and water, dried over $MgSO_4$ and the solvent evaporated. Crude product was purified by flash chromatography (*n*-hexane:ethyl acetate 8:1) to give the desired product **6** as white solid (180 mg, 48% yield). ^{1}H NMR ($CDCl_3$): δ= 5.26 (s, 2H; CH_2), 7.07 (d, J = 8.4 Hz, 2H, aromatic azide), 7.38 (d, J = 9.2 Hz, 2H, aromatic nitro), 7.44 (d, J = 8.4 Hz, 2H, aromatic azide), 8.28 (d, J = 9.2 Hz, 2H; aromatic nitro). MS: m/z: 353.7 $[M+K]^+$. Spectroscopic data for the title compound were consistent with the literature.[3]

2.3. Synthesis of precursor peptides (NH$_2$-**FFX**-OH)

Peptides were prepared manually via solid-phase peptide synthesis using classic Fmoc/tBu strategy. Wang resin was used as the solid support and the first Fmoc-amino acid was loaded manually using *N,N*-diisopropylethylamine (DIEA) in dichloromethane, following which the loading was controlled using UV measurement at 290 nm. Fmoc-protected amino acid residues were then coupled to the resin through HOBT/HBTU/DIEA activation method and Fmoc-deprotection was carried out using 20% piperidine in DMF. Following completion of the sequences, peptides were released from the resin with concomitant removal of protecting groups by treatment with TFA/TIPS/H_2O (95/2.5/2.5) for 3 hours at room temperature. The TFA was then evaporated and, depending on the sequence, the peptide was then either precipitated and washed with cold diethyl ether before being lyophilized, or diluted in a mixture of water and acetonitrile and lyophilized twice. Final purification was performed on a semi-preparative C18 column (Vydac). The resulting peptides were characterized by a combination of RP-HPLC on an analytical C18 column (Waters) and Mass Spectrometry using ESI as the ionization method.

2.4. Synthesis of azidobenzyl carbamate modified peptides (1-3)

To a solution of 4-azidobenzyl 4-nitrophenyl carbonate **6** (50 mg, 160 µmol, 1.25 eq) in 250 µL dichloromethane, 55 µL of DIEA was added and the solution cooled to 0 °C. A previously prepared solution of precursor peptide (128 µmol, 1 eq.) in 300 µL dimethylformamide was added dropwise over 2 minutes while keeping the temperature at 4 °C. After a further 55 µL of DIEA was added, the solution was allowed to warm up at room temperature and stirred overnight. The mixture was then diluted with 300 µL methanol and purified by semi-preparative HPLC to give the desired products. HPLC spectra of the final peptides **1**-**3** are reported in the following section.

3. Compounds Characterization

3.1. NMR Spectra of small molecules

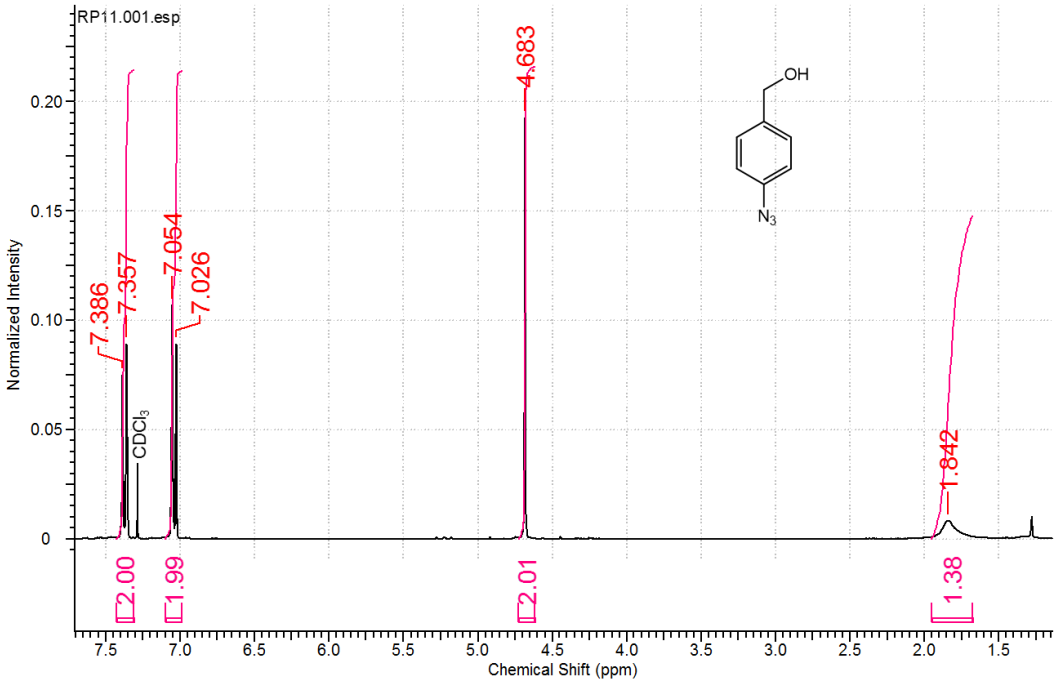


Figure S1. ^{1}H NMR spectrum of compound **5** (300 MHz, CD_3OD).

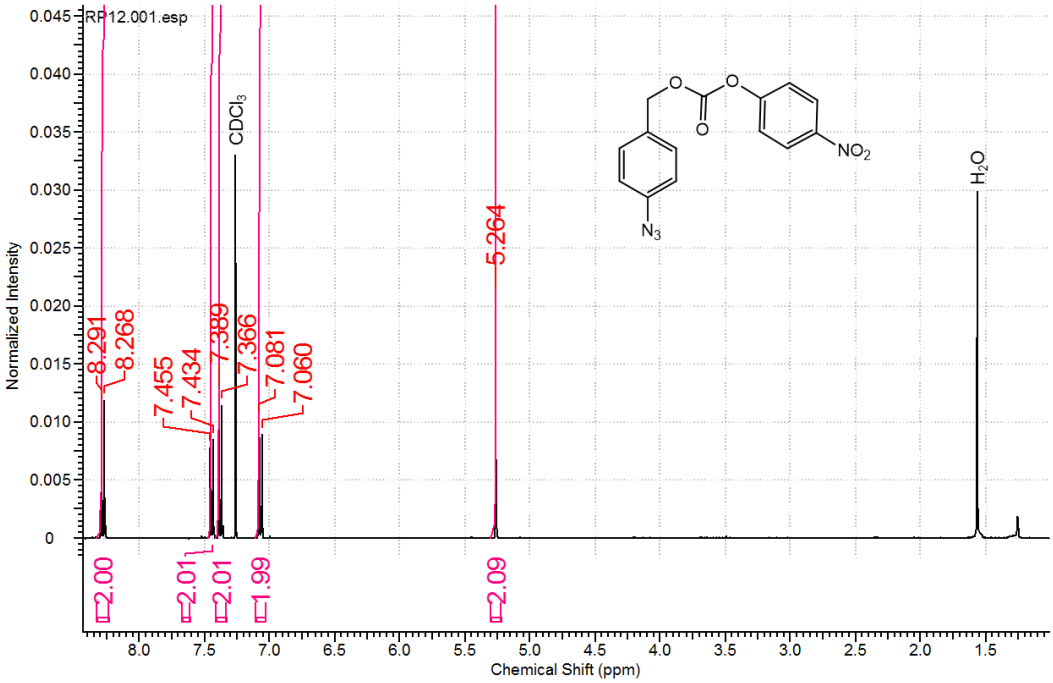
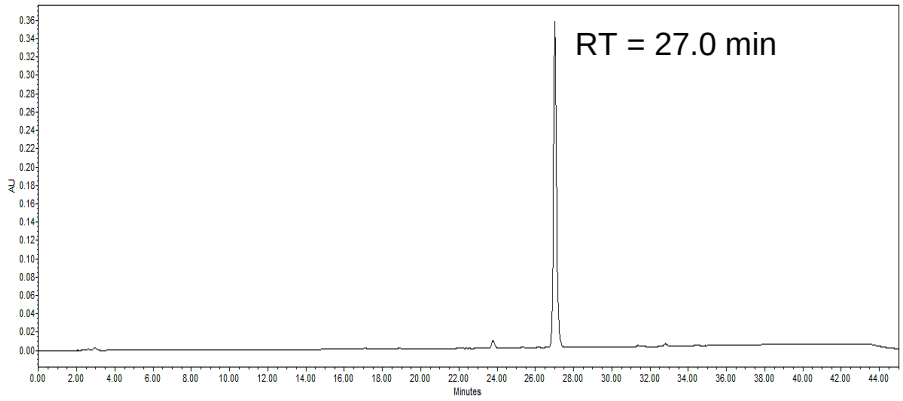


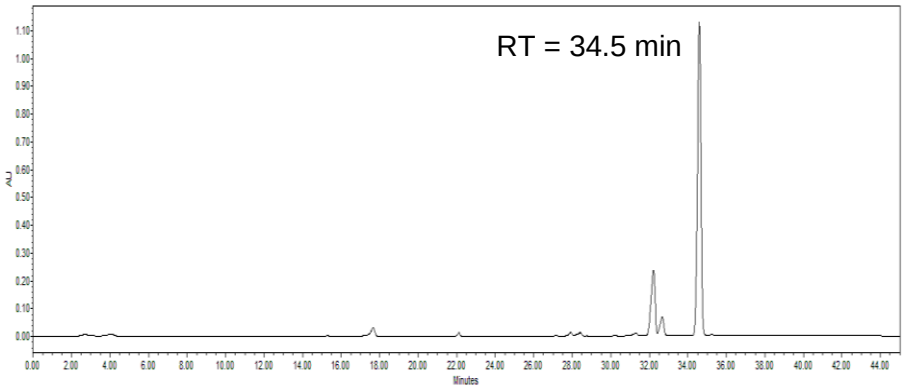
Figure S2. ^{1}H NMR spectrum of compound **6** (400 MHz, CD_3OD).

3.2. Peptide Characterization



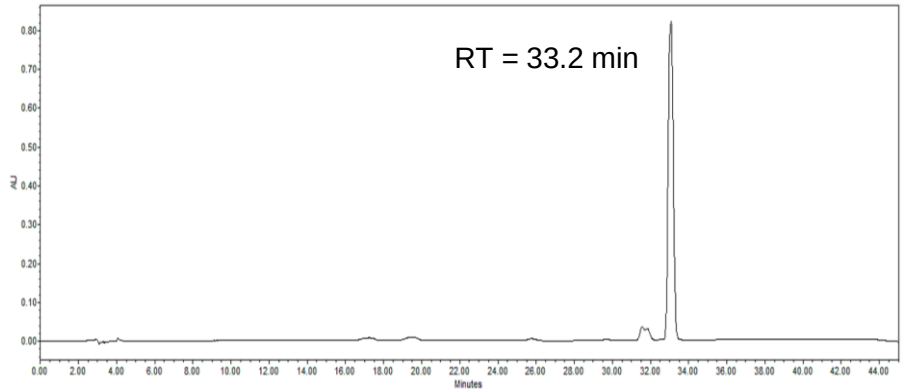
AzBz-[FFG]-OH: MW = 544.6 g.mol^{-1}; ***m/z* (ESI)** 545.5 $[M+H]^+$

Figure S3. HPLC spectrum at 254 nm and MS characterization of compound **1** (purity = 98%).



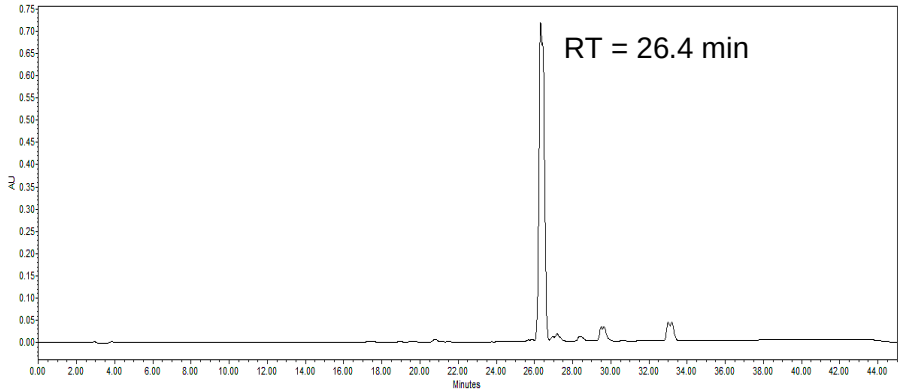
AzBz-[FFL]-OH: MW = 624.6 g.mol^{-1}; ***m/z* (ESI)** 625.7 $[M+Na]^+$

Figure S4. HPLC spectrum at 254 nm and MS characterization of compound **2** (purity = 80%).



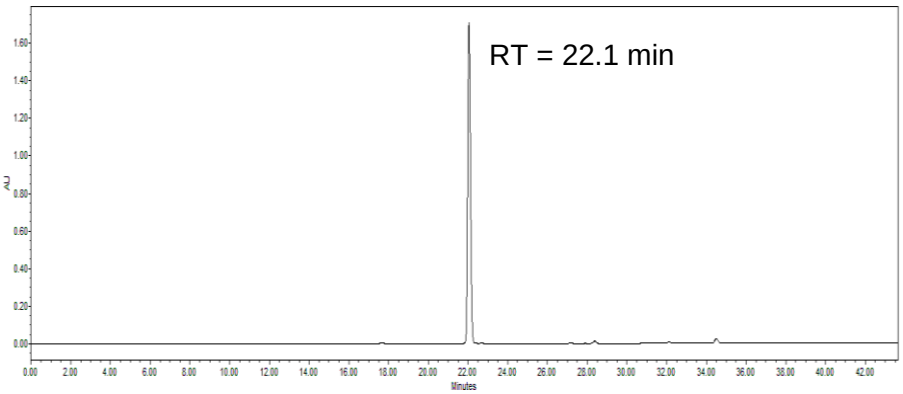
AzBz-[FFF]-OH: MW = 634.7 g.mol^{-1}; ***m/z* (ESI)** 635.8 $[M+H]^+$

Figure S5. HPLC spectrum at 254 nm and MS characterization of compound **3** (purity = 91%).



AzBz-[FFE]-OH: MW = 616.6 g.mol^{-1}; ***m/z* (ESI)** 639.6 $[M+Na]^+$

Figure S6. HPLC spectrum at 254 nm and MS characterization of compound **4** (purity = 89%).



AzBz-[FFR]-OH: MW = 643.6 g.mol^{-1}; ***m/z*** **(ESI)** 644.5 $[M+Na]^+$

Figure S7. HPLC spectrum at 254 nm and MS characterization of compound **5** (purity = 99%).

4. Additional Figures

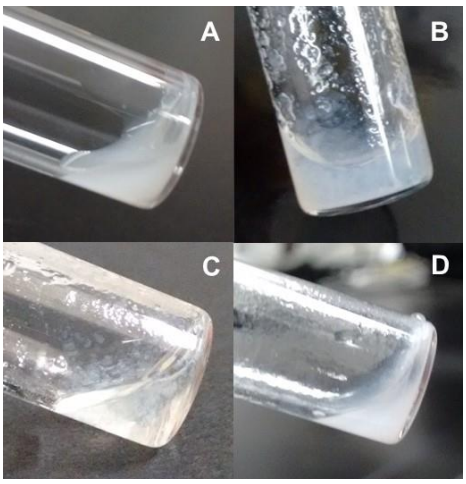


Figure S8. Results of gelation experiments for (A) AzBz-FFF, (B) AzBz-FFL, (C) AzBz-FFE and (D) AzBz-FFR at 0.5% wt in pure water + 5 % DMSO. In all four cases, no hydrogel formation was observed.

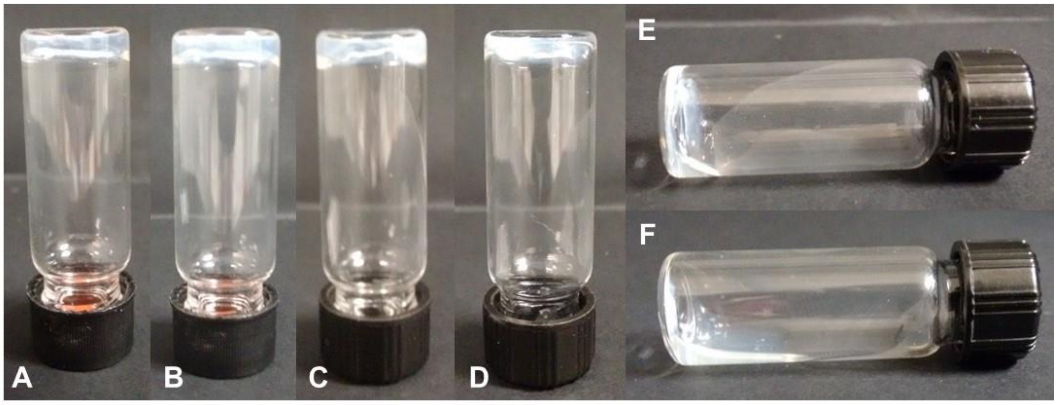


Figure S9. Effects of variations of the pH for a hydrogel of 0.5% wt of **1** in pure water + 5% DMSO. The pictures corresponds to hydrogel at a pH of (A) 4.7, (B) 6.2, (C) 7.0, (D) 8.1, (E) 9.0, (F) 10.0.

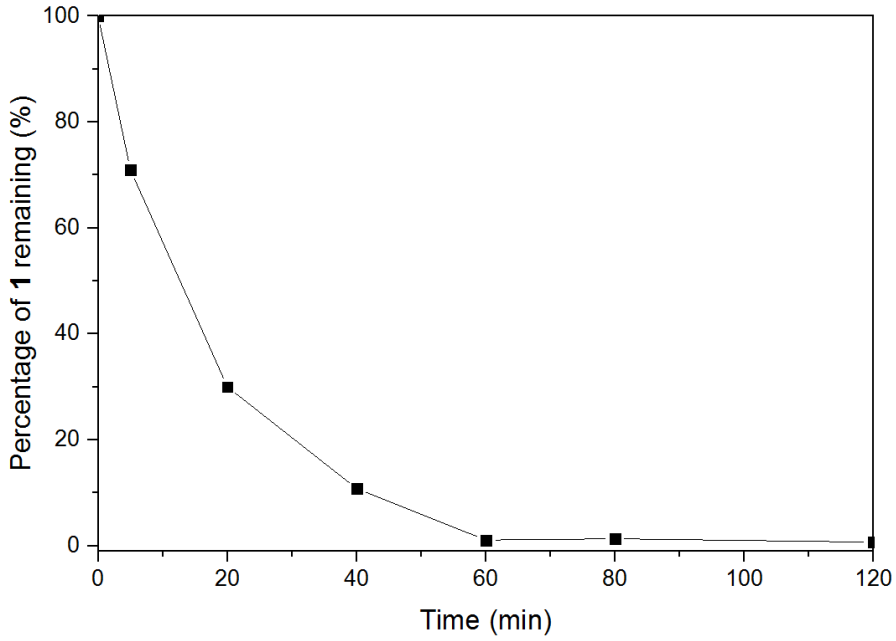


Figure S10. Degradation of a solution of peptide **1** (0.33 mM) in pure water + 5% DMSO over time, upon addition of 46 equivalents of H_2S in PBS (1 M, pH = 7.4) and incubation at 37 ℃. The percentage of peptide **1** remaining was characterized by HLPC analysis and calculated using the area of integration of the peak corresponding to **1** at 254 nm.

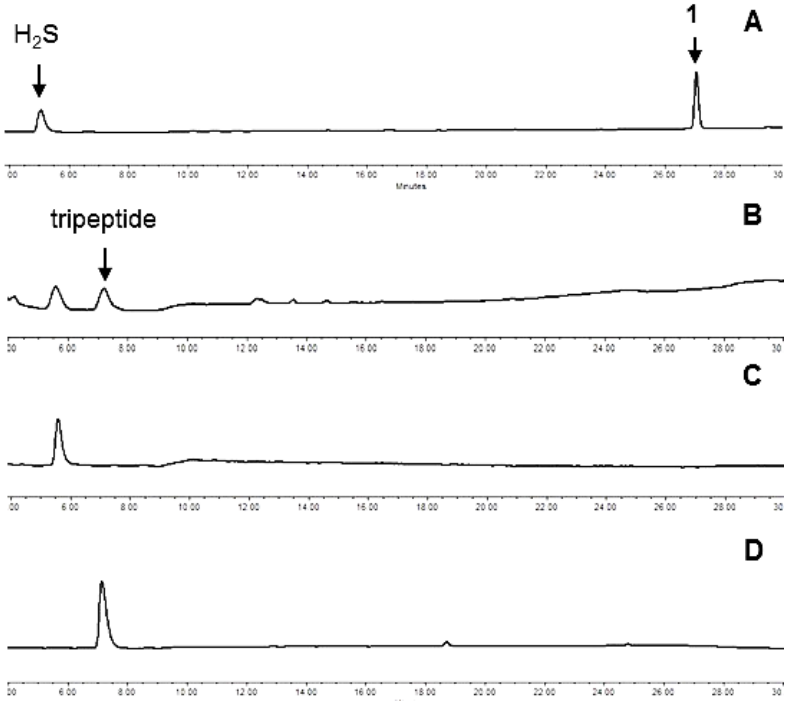


Figure S11. HPLC spectra at 220 nm of (A) a solution of peptide **1** in pure water + 5% DMSO in the presence of 46 equivalents of H_2S in PBS (1 M, pH = 7.4) at t = 0 and (B) after 2 hours incubation at 37 ℃; (C) a solution

of H_2S; (D) a solution of tripeptide FFG. The peaks for (A) peptide **1** and (B) tripeptide were isolated and further characterized by MS spectroscopy.

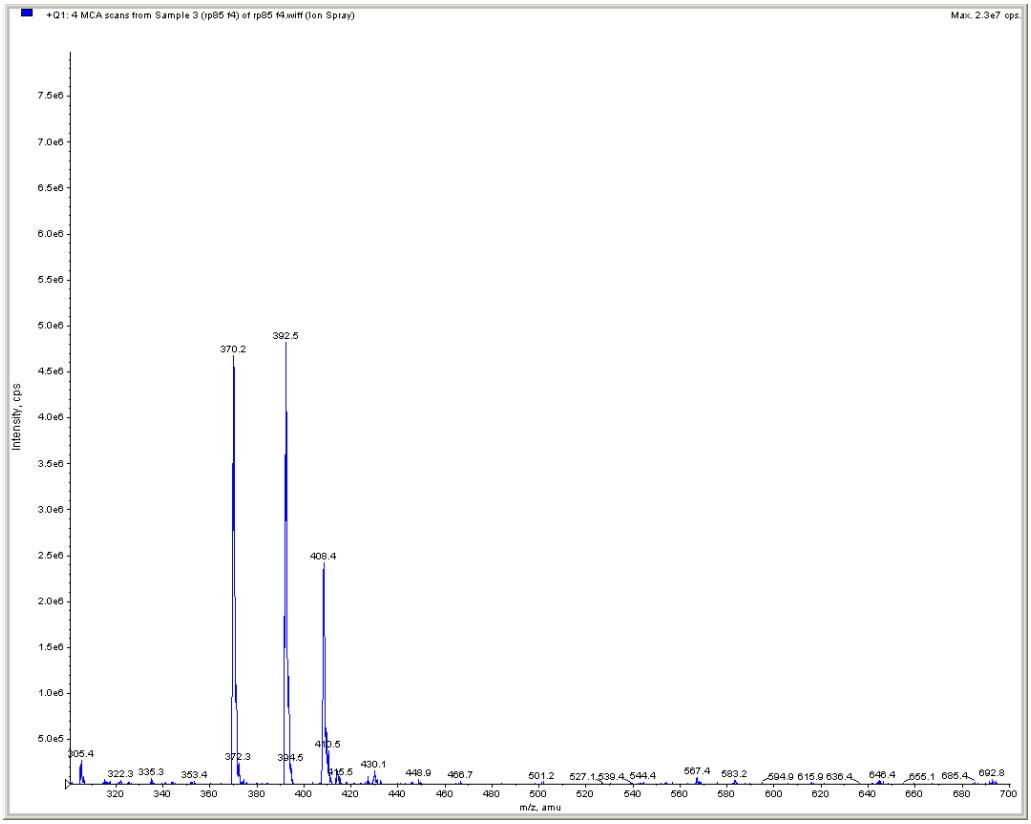


Figure S12. MS spectrum of peptide 1 after reacting with H_2S from HPLC fractions. Target Mw = 369.2; Observed: 370.2 $[M+H]^+$, 392.5 $[M+Na]^+$, 408.4 $[M+K]^+$.

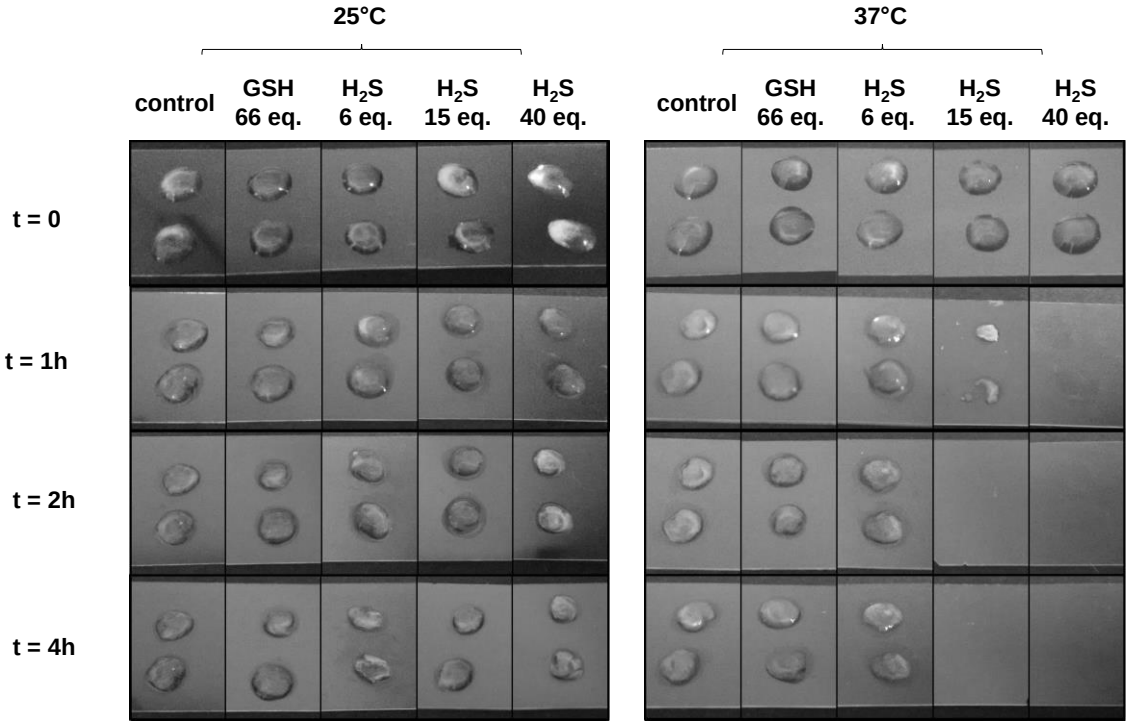


Figure S13. Photographs of peptide **1** hydrogel (0.4% wt) response to the addition of various equivalents of H_2S or GSH. The experiment was carried out in duplicate, on 20 µL hydrogel samples in pure water + 5% DMSO spotted on a glass plate. H_2S or GSH analytes were added in the form of 5 µL drops in PBS at pH = 7.4 at the appropriate concentration (0.18 M, 0.43 M, 1.22 M and 2 M for 6, 15, 40 and 66 equivalents respectively). Incubation was carried out at either 25 ℃ or 37 ℃ for the indicated amounts of time. Gels spots incubated with high levels of H_2S collapsed and were washed away during the washing step.

5. References

1. M. N. Hughes, M. N. Centelles and K. P. Moore, *Free Radical Biol. Med.*, 2009, **47**, 1346-1353.
2. E. A. Guenther, K. S. Johnson and K. H. Coale, *Anal. Chem.*, 2001, **73**, 3481-3487.
3. R. van Brakel, R. C. M. Vulders, R. J. Bokdam, H. Grull and M. S. Robillard, *Bioconjugate Chem.*, 2008, **19**, 714-718.