Supporting Information

Elastase-triggered H_2S delivery from polymer hydrogels

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Experimental section

Materials

Rink amide MBHA resin (0.754 mmol/g) and fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids were purchased from P3 Biosystems and used as received. HNE was purchased from Athens Research & Technology. Upon arrival, it was dissolved in water, aliquoted, and lyophilized, prior to being stored at -80 °C as a salt-free powder. Carboxymethyl cellulose (CMC, MW = 90,000, DS = 0.7) was purchased from Acros and used as received. Polyethylene glycol (HO-(PEG)-OH; MW = 600) was purchased from BeanTown Chemical. All other reagents were purchased from commercial vendors and used as received without further purification unless noted otherwise.

Dulbecco's Modified Eagle Medium (DMEM, VWR, Radnor, PA), 10% fetal bovine serum (FBS, VWR, Radnor, PA), penicillin/streptomycin (MP Biomedicals), and trypsin with 0.25% EDTA solution (VWR, Radnor, PA) were purchased from VWR.

Peptide synthesis

All peptides were prepared by solid phase peptide synthesis (SPPS) using a Liberty 1 peptide synthesizer with (hexafluorophosphate benzotriazole tetramethyl uronium) (HBTU)/ *N,N*-diisopropylethylamine (DIEA) chemistry. In general, to 1 equiv of MBHA resin amine, 4 equiv of Fmoc-amino acid, 3.6 equiv of HBTU and 8 equiv of DIEA were dissolved in DMF and added. The mixture was heated up to 75 °C via microwave heating for five minutes. The Fmoc group was deprotected with piperazine under similar microwave conditions. 4-Formylbenzoic acid (FBA) coupling was performed with the same HBTU/DIEA chemistry but at room

temperature and without the use of the microwave. In this coupling, 1 equiv of MBHA resin amine, 4 equiv FBA, 3.9 equiv HBTU and 8 equiv of DIEA were dissolved in DMF. The mixture was allowed to react on a shaker for 2 h, followed by washing with DMF (2×10 mL) and DCM (2×10 mL). After cleavage (97.5 vol% TFA and 2.5 vol% water, shaking for 2 h), the peptides were purified by preparative HPLC, with ACN/H_2O with 0.1 vol% TFA. Fractions were analyzed with an Advion ExpressIon compact mass spectrometer (MS), and product-containing fractions were combined and lyophilized. MS results of synthesized peptides are shown in Figure S1.

Synthesis of di-carboxylate PEG (HOOC-PEG-COOH)

HOOC-PEG-COOH was synthesized by oxidizing HO-PEG-OH with Jones reagent. Jones reagent was prepared by adding concentrated sulfuric acid (5 mL) to water (35 mL), followed by the addition of CrO_3 (6.64 g). The solution was orange. For the oxidation reaction, HO-PEG-OH (5 g, 16.6 mmol) was dissolved in acetone (60 mL). Next, the polymer solution was added to the Jones reagent solution (cooled on an ice bath) dropwise. The mixture was stirred for another 30 min with a color change from orange to green, after which the organic layer was blown away with air. The remaining mixture was extracted with CH_2Cl_2 (3 x 20 mL), followed by drying the organic layer with anhydrous $MgSO_4$. After rotary evaporation, the product was obtained as colorless viscous liquid. The reaction proceeded with quantitative conversion, which was confirmed by thin layer chromatography.

Safety warning: CrO_3 is toxic, carcinogenic and oxidative. Please refer to its SDS before use.

Hydrogel synthesis

The hydrogel with 35% CMC and FBA-VKVKVK-NH_2 was prepared as follows (Scheme S1). All other hydrogels were prepared in a similar way.

Peptide FBA-VKVKVK-NH_2 (200 mg, 720 μmol amine) was dissolved in PBS buffer (4 mL, 10 mM, pH = 7.4, applying to all PBS mentioned unless otherwise indicated), followed by adding CMC (85 mg, 252 μmol carboxylate, pre-dissolved as 20 mg/mL in PBS) and HOOC-PEG-COOH (145 mg, 468 μmol carboxylate) individually, and then vortexed for 2 min. *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC, 275 mg, 1440 μmol) and *N*-

hydroxysuccinimide (NHS, 165 mg, 1440 μmol) were then added, and the total volume was then diluted to 10 mL by adding PBS buffer (final pH = 5.0). The mixture was then vortexed for another 5 min. The hydrogel formed within 10 min. After reacting overnight without stirring, the hydrogel was crushed with a spatula, and Milli-Q water (20 mL) was added to the hydrogel, followed by 30-min sonication and vacuum filtration. This sonication-filtration procedure was repeated twice more to remove EDC and NHS, prior to lyophilization of the powder.

_S_-Benzoylthiohydroxylamine (SBTHA) coupling

SBTHA (100 mg, 654 μmol, prepared following literature procedures[36]) and dry dimethyl sulfoxide (DMSO, 15 mL) were added to the lyophilized hydrogel powder, followed by 2 min vortexing. Dowex resin (50 mg) and activated molecular sieves (~50 beads) were wrapped in a piece of filter paper, which was tied with a cotton thread, and this assembly was added to the solution. The mixture was allowed to react for 6 h without stirring at rt. CH_2Cl_2 (40 mL) was then added after removing the paper pack, followed by 30-min sonication and vacuum filtration. This sonication-filtration procedure was repeated twice more to remove unreacted SBTHA and DMSO. The final product was dried under vacuum and obtained as white powder.

H_2S-releasing profile analysis

A special glass vial with a small well attached at the bottom was used, similar to previous reports.[36,39] For the HNE-free release test, hydrogel powder (1 mg) was added into the well, along with PBS (100 μL). The vial was then sonicated for half an hour to ensure hydration. Cysteine (0.242 mg, 2 μmol, aliquoted from a stock solution in Milli-Q water and lyophilized) was dissolved in PBS (10 μL) and added to the well, which was immediately covered with a Breathe-EASIER membrane (Diversified Biotech) and sealed with an O-ring. PBS (10 mL) was added to the glass vial, along with a stir bar. The entire setup was immersed in a water bath at 37 ^{o}C, which was monitored using a hot plate with a feedback temperature probe (Corning). An H_2S-sensitive electrode probe (WPI) was submerged into the PBS, and H_2S concentration was monitored over time.

For H_2S-release experiments in the presence of HNE, hydrogel powder (1 mg) was added to the well, along with PBS buffer (90 μL, 10 mM, pH = 7.56 to ensure pH = 7.40 for the final mixture). The well was then sonicated for 30 min to ensure hydration. HNE (2.5 or 5 μg, aliquoted from a stock solution in Milli-Q water and lyophilized, corresponding to 1 or 2 arbitrary units of HNE, respectively) was first dissolved in NaOAc/NaCl buffer (10 μL, 50 mM, pH = 5.5 with 150 mM NaCl, applying to all sodium acetate buffers mentioned unless otherwise indicated) to ensure homogenization, and then added to the well. The subsequent steps, including adding cysteine solution and sealing the well, were identical to the HNE-free release tests.

For H_2S release profile measurements in experts with HNE, following similar procedures to those without, hydrogel powder (1 mg) was added to the gel-holding well, along with PBS (100 μL) and cysteine (0.242 mg, 2 μmol, aliquoted from a stock solution in Milli-Q water and lyophilized). After setting up the apparatus similar to the procedures above, H_2S release was monitored for 24 h at 37 ^oC. The PBS buffer (10 mL) inside the vial was then removed, along with the membrane and O-ring. HNE (5 μg, aliquoted from a stock solution in Milli-Q water and lyophilized) in NaOAc/NaCl buffer (10 μL) and cysteine (0.242 mg, 2 μmol in 10 μL PBS) were added to the well, prior to resealing the well with a membrane and O-ring. Fresh PBS buffer (10 mL) was then added into the vial, an H_2S-sensitive electrode probe was submerged into the PBS, and H_2S concentration was monitored over time. As a control experiment, NaOAc/NaCl buffer (10 μL) and cysteine (0.242 mg, 2 μmol in 10 μL PBS) were added to the well without HNE. The apparatus was set up in a similar way as described above. Signals from the electric probe were collected and recorded by LabScribe software, then converted to H_2S concentration (μM) by a calibration curve determined at 37 ^oC. All release profiles are reported as the average of triplicate experiments, and error bars indicate standard deviation.

Rheology

The rheology of hydrogel samples was determined by oscillatory rheology experiments with a TA 2000 rheometer. Frequency sweep measurements were carried out using stainless steel parallel plate geometries (d = 8 mm) at 1% strain, 1 mm gap, and frequency from 0.1-100 Hz. The samples were cut into round disks with diameters of 13 mm, and thicknesses of 2 mm. Results are reported as the average of triplicate experiments, and error bars indicate standard deviation.

Circular Dichroism (CD)

CD spectra of FBA-VKVKVK-NH_2 from 190-250 nm were recorded on a Jasco J-815 spectropolarimeter (JASCO, Easton, MD, USA) using a 1 mm path length quartz cuvette (Thermo Fisher Scientific). The peptide was dissolved in PBS buffer at a concentration of 0.25 mg/mL. The results are reported as the average of triplicate experiments.

HNE degradation test of peptide in solution

An HNE-peptide degradation test with peptide FBA-VKVKVK-NH_2 was performed by first dissolving HNE (2.5 μg) in NaOAc/NaCl buffer (10 μL), followed by the addition of peptide in PBS buffer (1 mg/mL, 100 μL). Next, the mixture was immersed in a water bath at 37 ^{o}C. Aliquots from the reaction mixture (10 μL) were analyzed at predetermined time points with a matrix-assisted laser desorption ionization−tandem time of flight mass spectrometer (4800 MALDI TOF/TOF; AB Sciex).

Cell culture

An adherent H9C2 line of rat embryonic cardiomyocytes (ATCC) was used in this study with 2D cell culture method. Complete DMEM media was prepared by supplementing base DMEM with 10% fetal bovine serum (FBS), 50 IU/mL penicillin, and 50 μg/mL streptomycin. H9C2 cells were cultured with complete DMEM at 37 °C in 5% CO_2 in air. The cultures were passaged after 70–80 % confluence was achieved. Cells were rinsed with PBS buffer (100 μL) three times, and then released with trypsin and 0.25% EDTA solution (VWR). The suspension of released cells was centrifuged at 1000 rpm for 5 min.

Cell Viability Assays

H9C2 cells (5000 cells per well) were plated in a 96-well plate with 180 μL complete DMEM media in each well. Doxorubicin hydrochloride (Dox, 1.2 mg) was dissolved in PBS (10.3 mL) to prepare a 200 μM Dox stock solution. Lyophilized hydrogel powder (1 mg) was swollen in PBS (781 μL) to make a hydrogel stock suspension (664 μM in SATO). Cys (25.6 mM) and HNE solutions (concentrations noted below) were freshly prepared with PBS and NaOAc/NaCl buffers, respectively.

After culturing for 24 h, cells were washed with PBS three times, and complete DMEM media (125 μL) was added to each well. Before addition of Dox, cells in each treatment group were pretreated with the following combinations of reagents: (a): **Dox**; No pretreatment; **Gel+Cys+HNE+Dox**: gel (166 μM SATO groups), Cys (640 μM), HNE (8 μg/mL); **Gel+Cys+Dox**: gel (166 μM SATO groups), Cys (640 μM); **HNE+Dox**: HNE (8 μg/mL); **Cys+Dox**: Cys (640 μM); **Gel+Dox**: gel (166 μM SATO groups); **Gel+HNE+Dox**: gel (166 μM SATO groups), HNE (8 μg/mL); **Cys+HNE+Dox**: Cys (640 μM), HNE (8 μg/mL). Cells were incubated for 1 h, then Dox in PBS (5 μL, 200 μM) was added to each well to make the final Dox concentration 5 μM. After incubation for 24 h, cells were washed three times with PBS and then treated with serum-free DMEM (100 μL) and Cell Counting Kit-8 solution (10 μL, CCK-8, Dojindo, Rockville, MD). After incubation for another 2 h to allow for development of the CCK-8 dye, absorbance was recorded at 450 and 650 nm using a BioTek Synergy Mix plate reader (BioTek, Winooski, VT). Mean values are reported, and error bars indicate standard deviation, and group comparisons were determined by a one-way analysis of variance (ANOVA) and Tukey-Kramer HSD tests (n=5, ns = not significant vs Dox; *= $p < 0.01$ vs Dox; # = $p < 0.01$ vs Gel+Cys+Dox)).

Elemental Analysis

Elemental Analysis results were obtained from Midwest micro lab via Schoniger combustion.

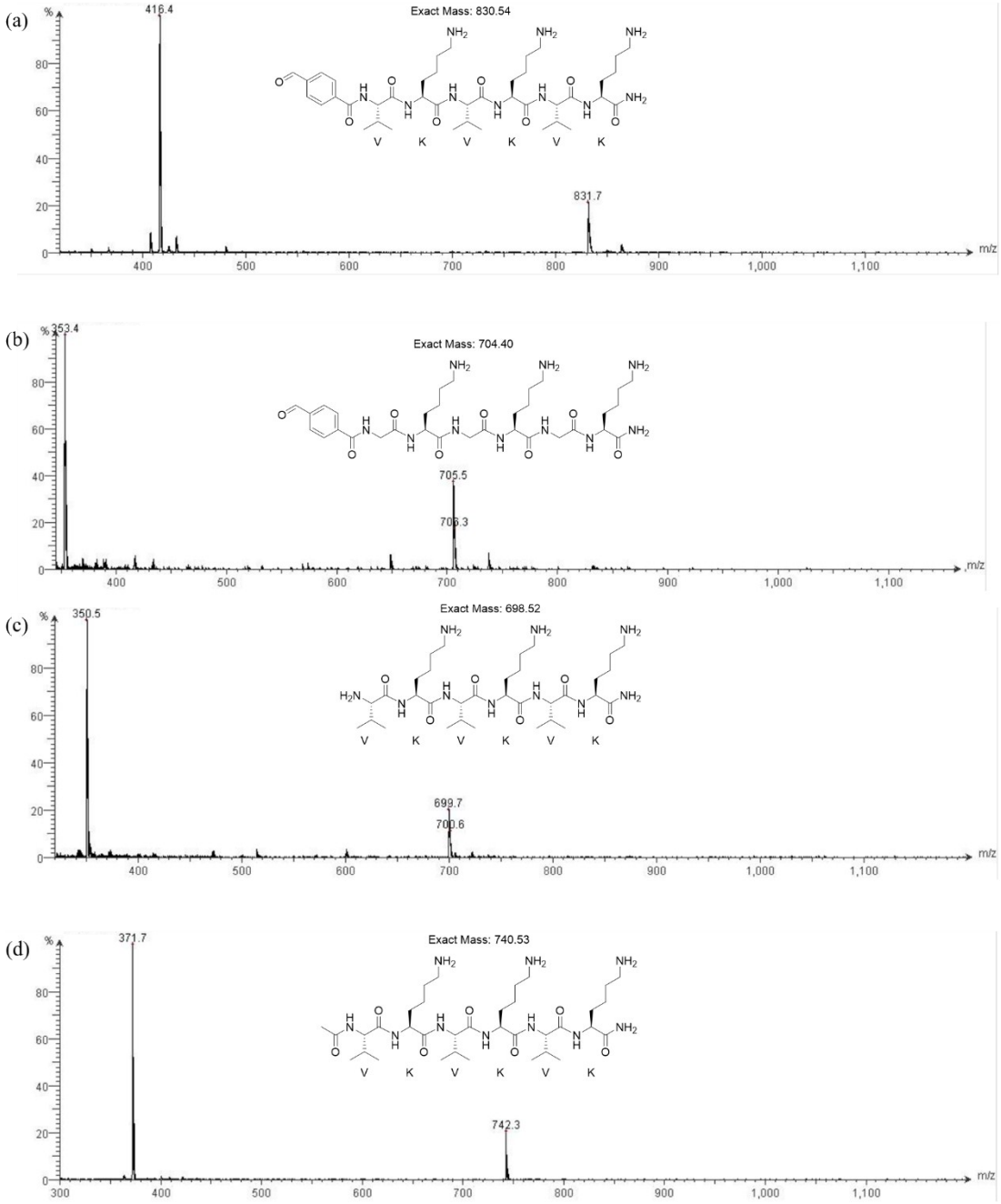


Figure S1 ESI-MS of (a) FBA-VKVKVK-NH_2, (b) FBA-GKGKGK-NH_2, (c) H-VKVKVK-NH_2 and (d) Ac-VKVKVK-NH_2.

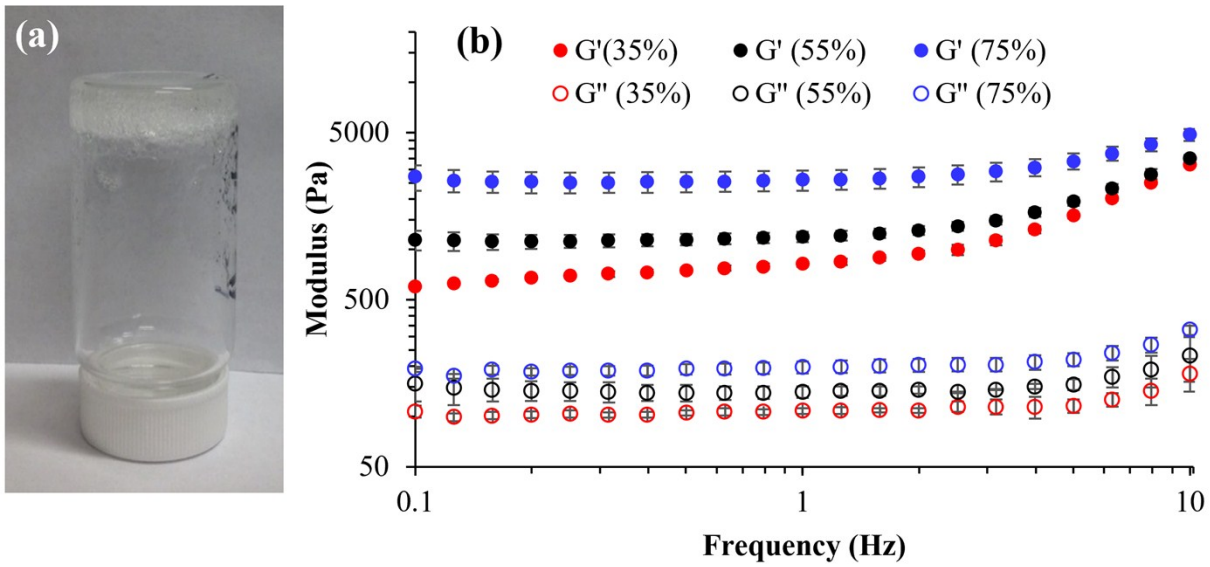
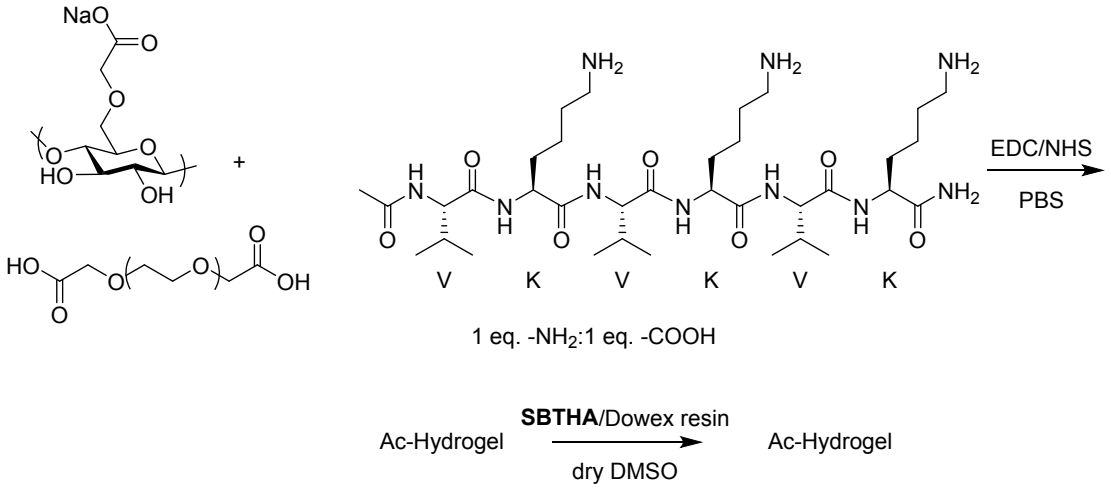


Figure S2. (a) Image of FBA-hydrogel with 35 % CMC and (b) frequency sweep trace of FBA-hydrogels with various CMC composition.

35% CMC	*Peaking H_2S concentration (μM)*	*Peaking time (min)*
No HNE	0.6±0.2	240±20
1 HNE	0.8±0.1	354±4
2 HNE	1.4±0.3	580±50

Table S1 Peaking concentrations and times for the H_2S-release curves at different concentrations of HNE.



Scheme S1. Synthetic route to the control Ac-hydrogel. To confirm that the sulfur from elemental analysis was from covalently attached SATO groups instead of physically encapsulated SBTHA or residual DMSO, we synthesized a control hydrogel by changing the aldehyde N-terminus to an acetamide group. SBTHA cannot react with the acetylated hydrogel or form the SATO group. Elemental analysis (sulfur) of the control Ac-hydrogel was 0.25 wt%, which is lower than the detection limit of 0.3 wt%. This result confirmed the successful formation of SATO-containing hydrogels and that any non-covalent SBTHA encapsulation was negligible.

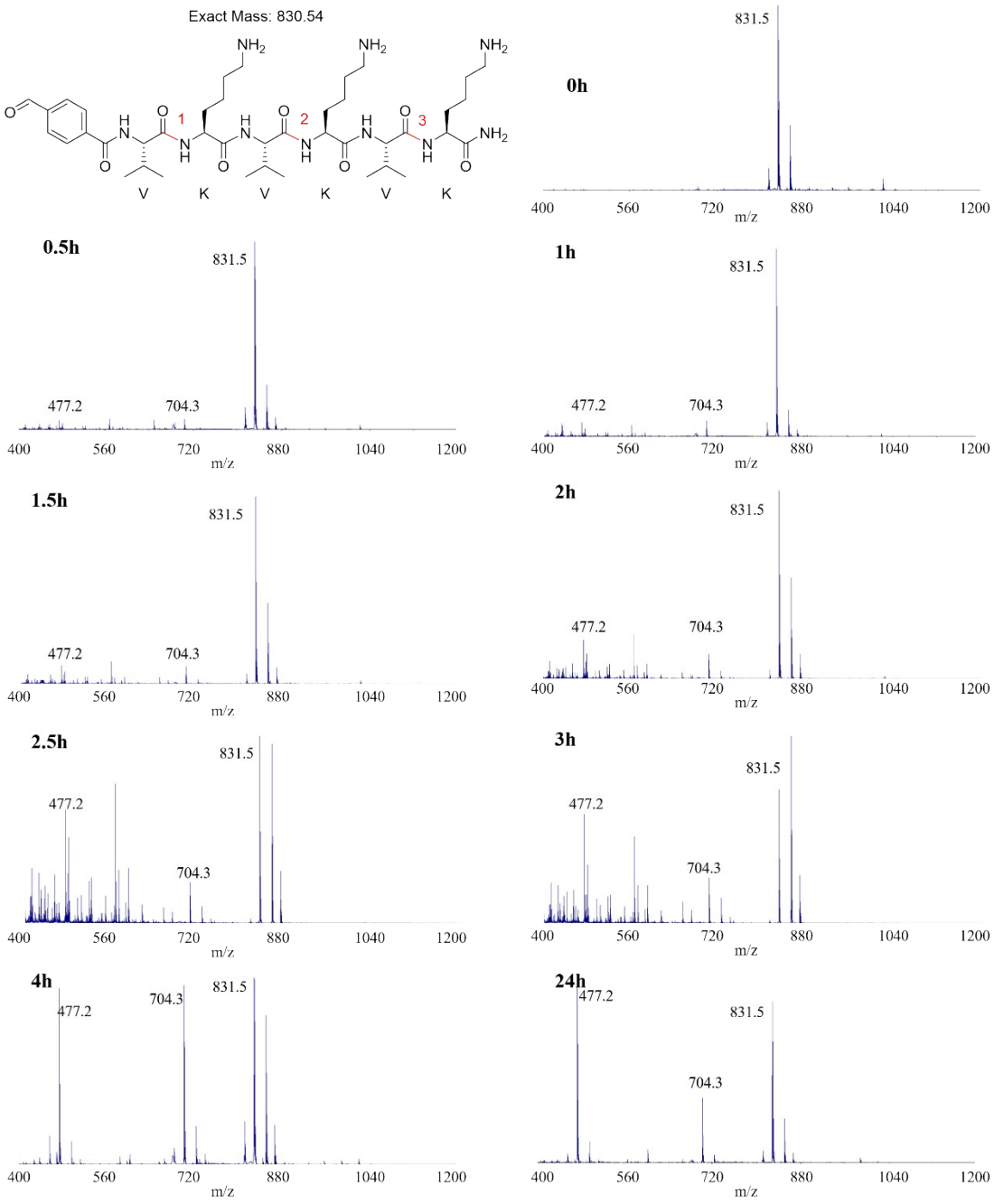


Figure S3 MALDI-TOF mass spectra showing degradation profile of peptide FBA-VKVKVK-NH_2 with 1 HNE in solution. The MW [M+H] of products cleaved at position 1, 2, and 3 is expected to be 600.5, 477.3 and 704.4, respectively (831.5 is the starting peptide). Peak 600.5 was never observed.

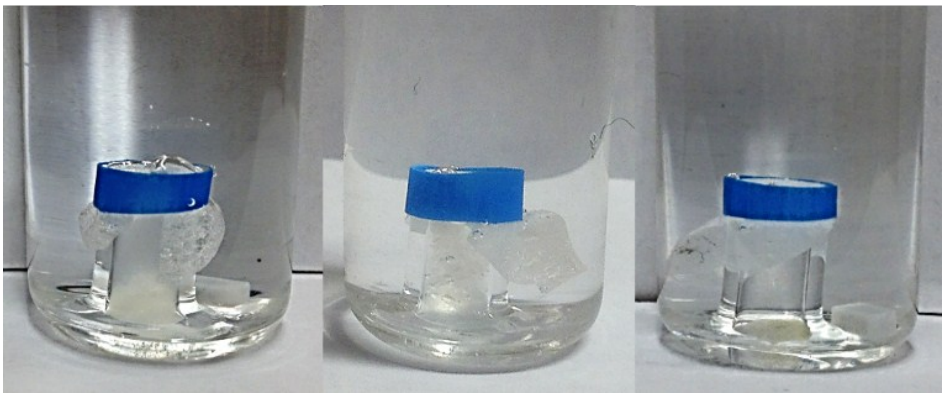


Figure S4. The H_2S-releasing apparatus and photos taken before the release test (left), after the release test without HNE for 24 h (middle, swelled) and after the release test with 2 HNE for 24 h (right, with most of the hydrogel degraded).

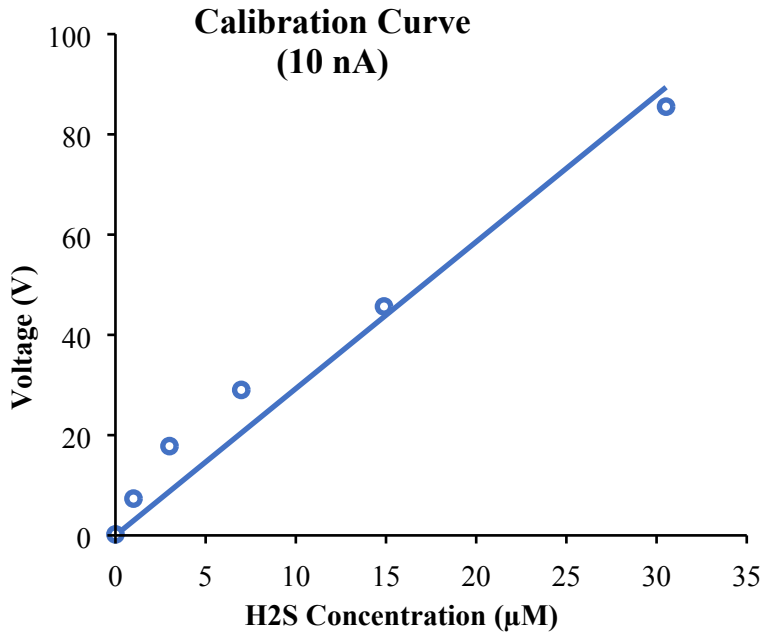


Figure S5 Calibration curve of probe voltage and H_2S concentration.

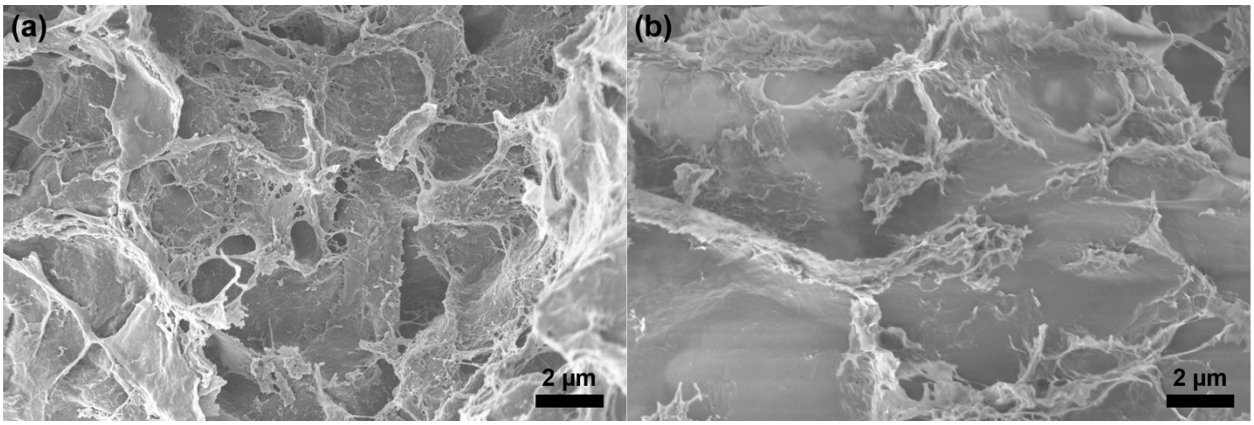


Figure S6 SEM images of (a) aldehyde-hydrogel and (b) SATO-hydrogel with 35% CMC and FBA-VKVKVK peptide.