

Supporting Information

Peptide-Based Hydrogen Sulfide-Releasing Gels

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Materials and Methods

Rink Amide MBHA resin and Fmoc-protected amino acids were purchased from ChemPep, Inc. All other solvents and reagents were purchased from commercial sources and used as received. H₂S fluorescent probe DT-OH was prepared and isolated as previously reported.¹

High-resolution mass spectra were taken on an Agilent Technologies 6230 TOF LC/MS mass spectrometer.

Purification by preparative-scale reverse phase-high performance liquid chromatography (RP-HPLC) was carried out on an Agilent Technologies 1260 Infinity HPLC system, eluting with a gradient of 2% ACN to 90% ACN in milliQ H₂O and using an Agilent PLRP-S column (100Å particle size, 25 x 150 mm) and monitoring at 220 nm. 0.1% NH₄OH was added to both mobile phases in the purification of **2**. MilliQ H₂O and ACN without any additives were used for the purification of **3**. Fractions were analyzed by mass spectrometry (Advion ExpressIon Compact Mass Spectrometer), and product-containing fractions were combined, rotovapped to remove ACN, and lyophilized (LabConco).

Analytical HPLC was performed on a Waters 2545 Binary Gradient Module and Waters SFO system, eluting with a gradient of 5% ACN to 55% ACN in water at a flow rate of 1.5 mL/min, using an XTerra RP C18 column (particle size 5 µm, 3 mm x 50 mm). Formic acid (0.1%) was added to both mobile phases. UV absorbance was monitored at 220 nm via Waters 2489 UV-vis Detector, and MS spectra collected on a Waters Zspray SQ Detector 2.

Circular dichroism spectroscopy was carried out using a Jasco J-815 CD spectrometer at room temperature with a constant N₂ flow of 120 mL/min. The range of wavelengths employed was 400-190 nm (50 nm/min) with a response time of 8 sec. Samples at 0.25 mg/mL were measured in a 1 mm path length quartz cuvette. Samples were freshly prepared and briefly sonicated before analysis. Samples at 10 mg/mL were run in a 0.2 mm cuvette, and data points with an HT value of >800 (below 205 nm) were removed. All measurements were conducted in triplicate.

FT-IR spectra were obtained on a Varian 670 FT-IR Spectrometer equipped with a diamond Specac Golden Gate attachment. All spectra were an average of 24 scans and were recorded from 4000 to 600 cm⁻¹. A background spectrum without sample was collected and subtracted from sample spectra.

TEM samples were drop cast from 1 wt. % solutions of peptide in milliQ H₂O onto carbon-coated copper 300 mesh TEM grids (Electron Microscopy Sciences). Samples were then stained with a 2% solution of uranyl acetate in water. Images were taken on a Philips EM420 TEM with a slow scan CCD camera.

All rheological studies were conducted on an AR-G2 rheometer at room temperature. All time sweeps were performed at 1 Hz with 0.5% strain using an ETC Disposable plate - 986680 (25 mm plate; 0.5 mm gap distance). 20 mM CaCl₂ (25 µL) was added to a 1 wt % solution of peptide in milliQ H₂O (250 µL), allowing it to gel on the rheometer. The reported storage modulus was taken from the time sweep at 60 min. Frequency sweep experiments were performed with a log ramp frequency of 0.01 – 100 Hz and a constant strain of 0.5%. Strain sweep experiments were performed with a constant frequency of 1 Hz and a log ramp strain of 0.1 – 100%. All rheological measurements were conducted in triplicate, and error bars represent the standard deviation.

Fluorescence spectroscopy was performed on a Varian Cary Eclipse Fluorescence Spectrophotometer with a scanning speed of 400 nm ∞ min⁻¹, a 1 nm data pitch, an integration time of 0.1 sec and 5 nm slits. Measurements of peptide **3** (λ_{ex} = 290 nm) were taken in a 1 cm quartz cuvette.

UV-Vis absorption spectra were recorded from 800 nm to 200 nm on a Varian Cary 100 UV-Vis spectrophotometer with a scanning speed of 400 nm min^{-1} with light source changeover at 400 nm. Measurements of peptide **3** were taken in water in a 1 mm quartz cuvette or a 0.2 mm quartz cuvette.

Dynamic light scattering (DLS) was conducted using a Malvern Zetasizer Nano operating at 25 °C. A solution of peptide **2** was prepared at 5 mg/mL and filtered with a 0.2 μm filter prior to scanning. The calculations of the particle size distributions and distribution averages were conducted using CONTIN particle size distribution analysis routines with intensity averages. Measurements were made in triplicate and errors reflect standard deviations.

Synthesis of Peptide 2

Rink amide MBHA resin (100-200 mesh; 0.48 mmol/g) was added to a peptide shaker vessel, and the resin was allowed to swell in dichloromethane (DCM) (10 mL) for 20 min prior to adding the first amino acid. A deprotection solution of 2% piperidine and 2% 1,8-diazabicycloundec-7-ene (DBU) in *N,N*-dimethylformamide (DMF) was used to remove the *N*-terminal Fmoc group. 10 mL of this solution was added to the reaction vessel and the reaction mixture was agitated for 5 min. The solution was removed and this step was repeated once more. The resin was washed with DMF (3 x 5 mL) and DCM (3 x 5 mL). A coupling solution of amino acid (4 equiv), *N,N,N,N*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluoro-phosphate (HBTU) (3.9 equiv), and *N,N*-diisopropylethylamine (DIEA) (0.6 mL, 6 equiv) dissolved in 15 mL of DMF was added to the reaction vessel containing the resin, and the suspension was agitated for 2 h. The solution was drained and the resin was washed with DMF (3 x 5 mL) and DCM (3 x 5 mL). This procedure was repeated for each coupling step. After deprotection of the last amino acid (Ile), the linker, 4-formylbenzoic acid (FBA), was introduced using standard amino acid coupling procedures as described above. Once the linker was successfully coupled to the peptide, the peptide was cleaved from the resin by treatment with trifluoroacetic acid (TFA)/H₂O (97:3) for 2 h under constant agitation. The cleavage solution containing the peptide was drained into a 250-mL round bottom flask, and the resin was washed with DCM (2 x 10 mL). The combined cleavage solution and washes were concentrated to 2 mL via rotary evaporation, and diethyl ether was added to precipitate out the peptide. The crude peptide was recovered via vacuum filtration, allowed to dry, and was stored at 4 °C until HPLC purification. To dissolve the peptide after cleavage, 0.1% NH₄OH in milliQ water was added and concentrated NH₄OH was added dropwise to adjust the pH to 8. The solution was passed through a 0.45 μm filter prior to HPLC purification. HRMS: *m/z* 820.3723 (820.3723 Calc *m/z* for [M+H⁺]), 843.3577 (843.3573 Calc for [M+Na⁺]), 858.3233 (858.3282 Calc for [M+K⁺]).

Synthesis of Peptide 3

To a solution of peptide **2** in DMSO (50 mg/mL) was added *S*-benzoylthiohydroxylamine (3 equiv), prepared as previously described.² TFA (3 equiv) and activated 3 Å molecular sieves were added to the solution, as a catalyst and a drying agent, respectively. After 20 min, the reaction mixture was precipitated into dry DCM (20 mL). The precipitate was vacuum filtered and placed under high vacuum to remove any additional DCM. Once dry, the crude product was stored in the freezer until purification. Just before purification, the crude product was taken up in milliQ water with 3 equiv K₂CO₃, which afforded an aqueous solution at pH ~7. This solution was passed through a 0.45 μm filter prior to HPLC purification. HRMS: *m/z* 953.3713 (953.3720 Calc for [M+H⁺]).

Determination of critical micelle concentrations (CMCs)

Stock peptide solutions were prepared in milliQ water with 0.1 mg/mL Nile Red at 10 mg/mL peptide. This solution was diluted with water + 0.1 mg/mL Nile Red to afford concentrations of 7.5 mg/mL, 5 mg/mL, 2.5 mg/mL, 1 mg/mL, 0.1 mg/mL, 0.01 mg/mL, 0.001 mg/mL, and 0.0001 mg/mL peptide. Fluorescence emission spectra were recorded for each sample ($\lambda_{\text{ex}} = 550 \text{ nm}$), and the intensity measured at 628 nm for each sample was plotted against the concentration. The CMC value was calculated by first

constructing a plot of fluorescence intensity vs. log[concentration]. The CMC value was taken to be the intersection between the linear fits of the high and low concentration regimes.

Calibration of H₂S Selective Probe

An EDTA solution was prepared at 150 μ M. The solution was purged vigorously with nitrogen for 20 min. Anhydrous Na₂S was added to a vial under inert atmosphere, followed by 20 mL of the EDTA solution (to make 5 mM H₂S). A small stir bar was added to a scintillation vial containing 20 mL of PBS buffer. The vial was placed on a stir plate. The H₂S sensor was immersed in the solution and the background current was allowed to stabilize for several minutes. Five aliquots of the H₂S solution were injected sequentially into the vial (20 μ L, 40 μ L, 60 μ L, 80 μ L, 100 μ L). The current increased rapidly after each injection before reaching a plateau. The second aliquot was injected as soon as the current had stabilized. The other aliquots were injected similarly. The recorded data was used to construct a linear calibration curve of concentration vs. current.

Hydrogen Sulfide Release Experiments

A stock solution of 20 mM Cys was prepared in PBS buffer (1X, pH 7.4). A 20 mL scintillation vial equipped with a stir bar was charged with 3.8 mL of a 100 μ M solution of **3** in PBS buffer. An H₂S-selective microelectrode (World Precision Instruments) was submerged into this solution, and the output current was allowed to equilibrate for several minutes. Once a stable current was observed, 200 μ L of the Cys solution was added to the vial for a final concentration of 1 mM. The current was monitored for approximately 5 h. The vial was not stirred during analysis. No background subtraction was performed. A plot of H₂S concentration vs. time was constructed using the calibration curve, and the data were smoothed using a Savitsky-Golay filter in Origin.

To analyze release of H₂S from peptide **3** in gel form, 40 μ L of a 1 % wt. solution (~10 mM) of **3** was placed in a 1 dram vial. 20 mM aqueous CaCl₂ (4 μ L) was added to induce gelation. After 5 min PBS buffer (3.94 mL) was added to the vial, and the microelectrode was submerged into the solution. After the output current stabilized, 20 μ L of a 200 mM Cys solution was added to the vial for a final Cys concentration of 1 mM. The current was monitored for approximately 15 h. No background subtraction was performed. A plot of H₂S concentration vs. time was constructed using the calibration curve, and the data were smoothed using a Savitsky-Golay filter in Origin.

Cytotoxicity

Cytotoxicity measurements were performed using a live/dead assay (calcein AM and ethidium homodimer) on C57BL/6 mouse primary brain microvascular endothelial cells (Cell Biologics). Each experiment was repeated three times with n=5 for each run, and error bars reflect the standard deviation. Cells were passaged using standard cell culture procedures. Before plating, cells were detached with trypsin and centrifuged (2000 rpm for 10 min). The supernatant was removed, and the cells were resuspended in 1 mL complete endothelial cell media (Cell Biologics), and counted. Cells were plated in a 96-well plate at a concentration of 10,000 cells/well in a 96-well plate and incubated overnight (37 °C, 5% CO₂). Peptide **3** was dissolved in PBS at 80 μ M. The peptide solution was diluted with basal media containing 1 mM Cys to obtain peptide concentrations of 80 μ M, 60 μ M, 40 μ M, 20 μ M, and 2 μ M. Control wells contained basal media or basal media with 1 mM Cys. After incubating for 24 h (37 °C, 5% CO₂), the media was replaced with live/dead solution (made per the manufacturer's instructions), and the plate was left to incubate for 15 min. Cells were imaged and counted on a Nikon Eclipse Ti-U fluorescent microscope.

Cytotoxicity measurements for the peptide in gel form were carried out using the same live/dead stains as above. Using the same passaging and plating protocol as above, cells were seeded at 20,000 cell/well in a 48-well plate and incubated overnight (37 °C, 5% CO₂). Before gels were added, the media was removed and replaced with basal media or basal media with Cys (1 mM). Peptide gels were prepared in Slide-A-

Lyzer dialysis cups (2000 MWCO, Thermo Scientific) at 1% w/v by adding 30 μL peptide **3** to the cup followed by the addition of 3 μL of 0.2 M CaCl_2 . 10 min after the addition of CaCl_2 , the cups were placed in wells, and 50 μL of basal media (with or without Cys) was added to cover the gel. After incubating for 24 h (37 $^\circ\text{C}$, 5% CO_2), the cups and media were removed from the wells, and live/dead solution was added. The plate was left to incubate for an additional 15 min before imaging as above.

Fluorescence Probe studies of H_2S uptake *in vitro*:

Detection of H_2S uptake *in vitro* was analyzed using a turn-on fluorescent probe selective for H_2S (**DT-OH**). C57BL/6 mouse brain endothelial cells (Cell Biologics) were plated at 5,000 cells/well in 100 μL complete media per well and incubated at 37 $^\circ\text{C}$ and 5% CO_2 for 48 h. After aspiration of the media, the cells were washed with PBS and incubated with the probe (**DT-OH**) (5 μM) in PBS. After 20 min, the media was removed by aspiration, and peptide **3** (100 μM in PBS) with (+) or without Cys (-) 500 μM was added to the cells, and plates were incubated for an additional 20 min. Solutions were removed and replaced with PBS for imaging by fluorescence microscopy.

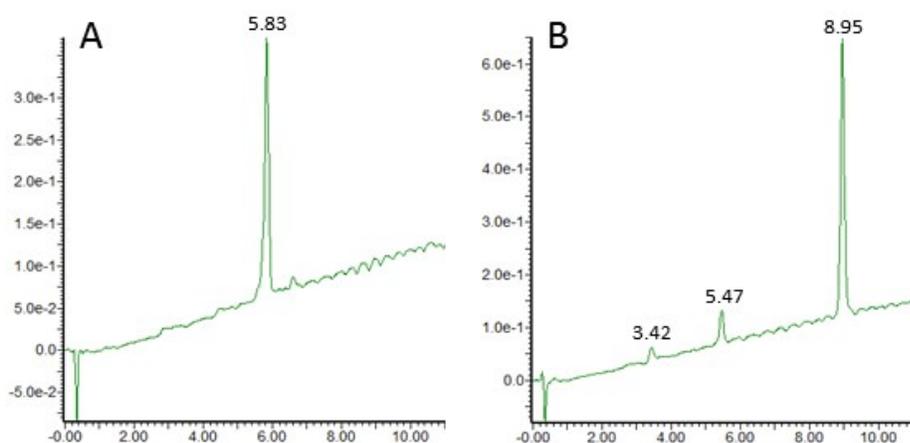


Figure S1. Analytical HPLC of peptides **2** (A) and **3** (B). The peaks at 3.42 and 5.47 min in the trace of peptide **3** are a result of hydrolysis under processing and HPLC conditions and correspond to *S*-benzoylthiohydroxylamine (**1**) (see Figure S2) and peptide **2**, respectively. Hydrolysis of the *S*-aroylthiooxime has been observed previously.^{2b}

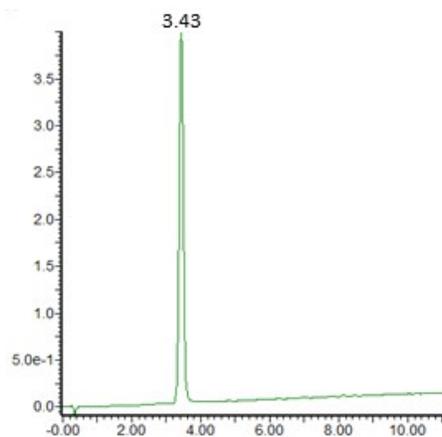


Figure S2. Analytical HPLC of *S*-benzoylthiohydroxylamine (**1**).

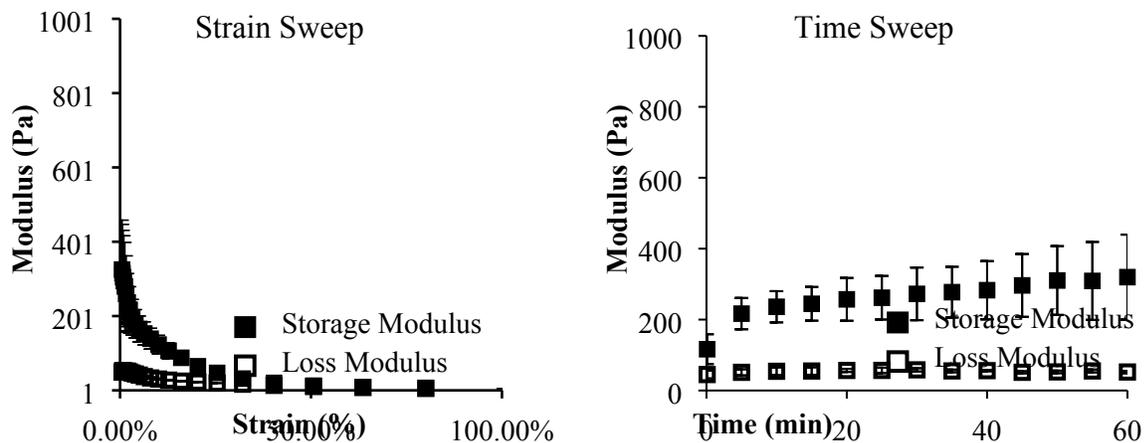


Figure S3. Strain sweep and time sweep rheological measurements of peptide **3**.

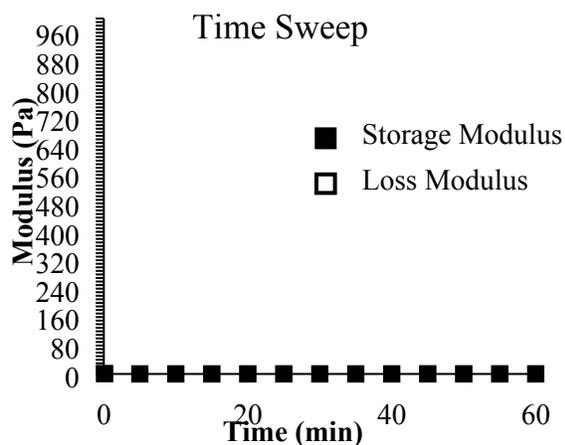


Figure S4. Time sweep rheology of peptide **2**.

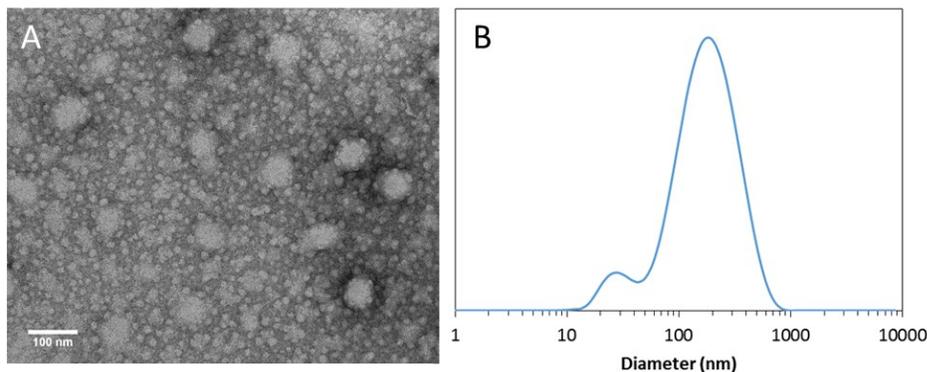


Figure S5. A) Conventional TEM of peptide **2** (cast from 10 mg/mL solution of peptide **2** in water). B) DLS measurements of peptide **2** (5 mg/mL in water). The large spherical structures in the TEM image appear to be compound micelles. DLS data are consistent with the TEM data, showing a population of small, spherical micelles with a diameter of 28 ± 8 nm as well as a population of larger structures with a diameter of 200 ± 110 nm.

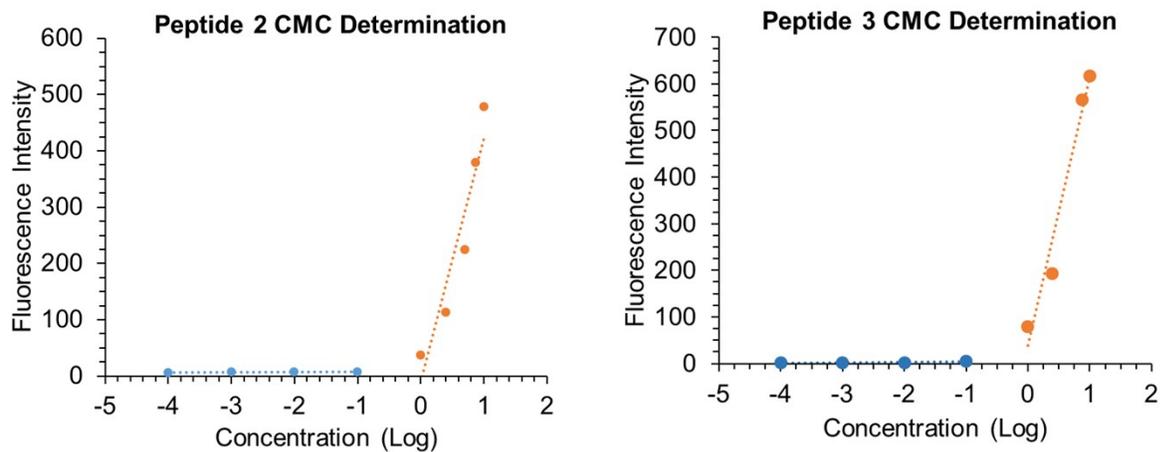


Figure S6. CMC data for peptides 2 and 3.

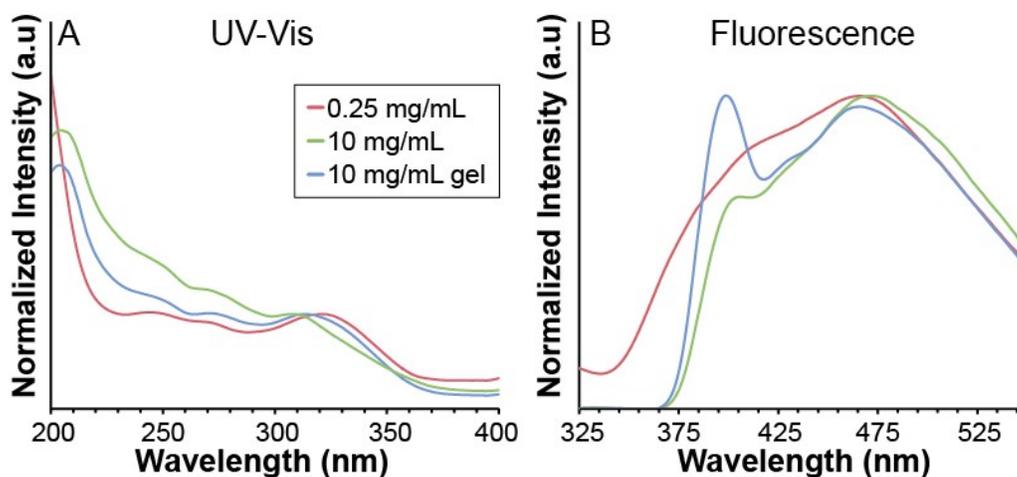


Figure S7. UV-vis (A) and fluorescence (B) spectroscopy of peptide 3. The emission peak at 398 nm that increases in intensity upon gelation is tentatively attributed to a higher prevalence of stacking for one of the aromatic rings upon aggregation and gelation. However, more extensive investigations are needed to fully describe the packing arrangement of the aromatic SATO groups.

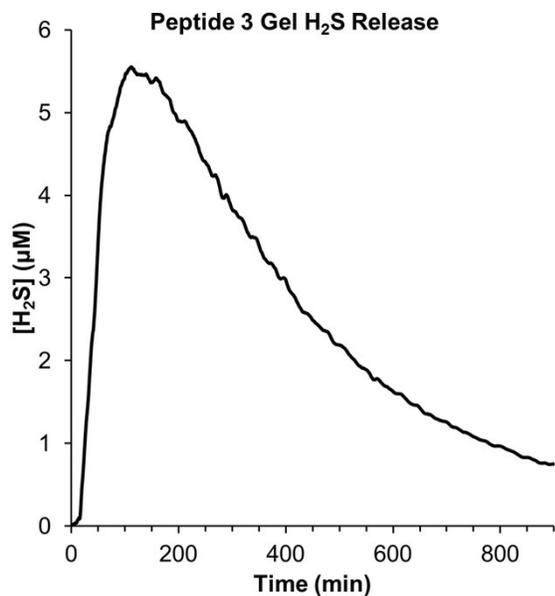


Figure S8. Release of H₂S from peptide gel **3**.

References

1. a) Shi, D. T.; Zhou, D.; Zang, Y.; Li, J.; Chen, G.-R.; James, T. D.; He, X.-P.; Tian, H. *Chem Commun.* **2015**, *51*, 3653. b) Yu, C.; Li, X.; Zeng, F.; Wu, S. *Chem Commun.* **2013**, *49*, 403.
2. a) Raasch, M. S. *J. Org. Chem.* **1972**, *37*, 3820. b) Foster, J. C.; Powell, C. R.; Radzinski, S. C.; Matson, J. B. *Org. Lett.* **2014**, *16*, 1558.