

**Peptide-Based Hydrogen Sulphide-Releasing Gels**

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Peptide-Based Hydrogen Sulphide-Releasing Gels

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An aromatic peptide amphiphile was designed for delivery of the signaling gas H₂S. The peptide self-assembled in water into nanofibers that gelled upon charge screening. The non-toxic gel slowly released H₂S over 15 hours, and the presence of H₂S in endothelial cells was verified using a fluorescent H₂S probe.

Despite its reputation as a foul-smelling, toxic pollutant, hydrogen sulphide (H₂S) is a vital biological signaling gas (gasotransmitter) produced endogenously by various enzymes.¹ Within the past few years, efforts devoted to studying the biological roles of H₂S, the enzymes that make it, and its therapeutic potential have expanded rapidly,² along with related efforts to improve its detection in vitro and in vivo.³ While many biological studies on H₂S are conducted using sulphide salts (NaHS and Na₂S), injections of aqueous sulphide solutions lead to a rapid surge of H₂S in the bloodstream followed by a rapid decline. To combat this uncontrolled delivery and more closely mimic the slow and sustained release of endogenous H₂S, our group and others have reported on small molecules that release H₂S with controllable kinetics.⁴ However, despite this progress on small molecule H₂S donors, very few efforts have been made in the area of H₂S-releasing materials.⁵

In addition to H₂S, nitric oxide (NO) and carbon monoxide (CO) are also classified as gasotransmitters. The study of NO biology has benefitted greatly from NO-releasing materials, including polymers, gels, microparticles, and others.⁶ The localized release provided by these materials allows for site-specific delivery of the gas, which minimizes the required dosage and off-target effects that can result from systemic NO administration. In fact, NO-releasing materials have been studied widely in preclinical animal models for various applications.⁷ Additionally, CO releasing materials have also been reported, although they have not yet reached the stage of animal models.⁸ In contrast, few H₂S-releasing materials

have been prepared for potential use in therapy: two polymeric H₂S donors⁵ and one H₂S-releasing metal-organic framework.⁹ While polymers and other nanoparticle drug delivery systems may be useful for targeting specific tissues through systemic administration, H₂S-releasing gels that can be localized at a site of interest are better suited as injectable biomaterials capable of filling a cavity or encapsulating cells while delivering precise doses of H₂S to an area of interest.

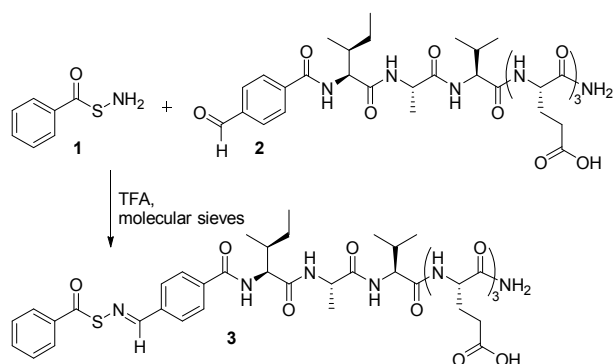
We recently reported on a family of small molecule H₂S donors based on the *S*-aroylthiooxime (SATO) functional group, which releases H₂S in response to thiol functionality.^{4e} SATOs are prepared by reaction of an *S*-aroyl thiohydroxylamine (Ar-C(O)S-NH₂) (SATHA) with an aromatic aldehyde or ketone. H₂S release is triggered by thiols such as cysteine, and release rates can be tuned by changing substituents on the SATHA ring, with half-lives of release in the range of 8-80 minutes.

In this report, we describe the preparation of a self-assembling peptide designed to form an H₂S-releasing gel. Self-assembling peptide-based materials include a wide range of peptide motifs that form one-dimensional nanostructures in aqueous solution.¹⁰ Gel formation in the range of 0.5-2 wt. % peptide can often be triggered by charge screening through physical entanglement of the extended nanostructures by pH changes or addition of salt. No chemical crosslinking is required, allowing for in situ gelation of the materials and shear-thinning behaviour. These qualities, along with their inherent biodegradability and minimal immunogenicity in most cases, make peptide-based gelators an attractive alternative to traditional polymeric hydrogels that require chemical crosslinking.

One class of self-assembling peptides is the aromatic peptide amphiphiles.¹¹ Comprised of short oligopeptides with terminal aromatic components, these small molecule gelators can be designed to self-assemble in water into extended cylindrical micelles or nanofibers.¹² The aromatic groups in aromatic peptide amphiphiles have traditionally served a structural role—none have included reactive groups designed to release a bioactive small molecule such as H₂S.

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Scheme 1. Synthesis of SATO-containing aromatic peptide **3** from SATHA **1** and aldehyde-containing peptide **2**.

Our design (Scheme 1) takes advantage of the inherent hydrophobicity of the SATO moiety to serve as both a structural component to drive self-assembly as well as a reactive functional group to release H_2S . We envisioned that a SATO-functionalized aromatic peptide amphiphile could be designed to form 1-dimensional nanostructures capable of gelation. Additionally, we anticipated that the SATO functional groups would be buried in the hydrophobic core of the nanostructures, thereby limiting their access to the cysteine trigger required for decomposition and potentially extending their H_2S -releasing capacity over hours to days.

The peptide design we envisioned included a hydrophobic SATO group attached to a short peptide that facilitated assembly into 1-dimensional nanostructures. In addition to aromatic stacking interactions in the nanofiber core, many aromatic peptide amphiphiles also rely on β -sheet-type hydrogen bonding along the long axis of the nanostructure to stabilize the cylindrical assembly.¹³ Toward this end, the peptide IAVE₃ was synthesized under standard solid phase conditions using Fmoc chemistry. The IAV region was designed based on the propensity of certain residues to self-pair in β -sheets found in natural proteins.¹⁴ Ala, Ile, and Val exhibit the highest tendency to self-pair; therefore, we incorporated these amino acids into the peptide sequence. The EEE sequence was included to provide the necessary hydrophilic/hydrophobic balance to achieve amphiphilicity. 4-Formylbenzoic acid (FBA) was added on resin under the same conditions as typical amino acid couplings, giving the modified peptide FBA-IAVE₃ (**2**). Following cleavage and purification, peptide **2** was allowed to react with SATHA **1** in the presence of TFA, employing molecular sieves as a drying agent. Full conversion to peptide **3** was observed after 20 min by ¹H NMR spectroscopy, and precipitation of the reaction mixture into CH_2Cl_2 afforded peptide **3**, which was purified by preparative HPLC.

Transmission electron microscopy (TEM) was used to characterize the morphology of the self-assembled architecture of peptide **3** (Figure 1A). Conventional TEM images revealed long, one-dimensional nanostructures with an average diameter of 10 nm. In contrast, TEM of peptide **2** (Figure S5) revealed predominately small spherical micelles around 10 nm in diameter and some larger spherical structures

around 50 nm in diameter. No one-dimensional objects were observed.

Next, we examined whether peptide **3** was capable of forming a robust gel. Addition of aqueous $CaCl_2$ solution to a 1 wt. % solution of peptide **3** in water led to instantaneous gelation (Figure 1B inset). As expected based on the TEM observations, addition of $CaCl_2$ to a solution of peptide **2** did not result in gelation. The viscoelastic properties of the self-assembled peptide hydrogel prepared from a 1 wt. % aqueous solution of peptide **3** were characterized by rheology (Figure 1B). These experiments revealed that the gel was robust, with a storage modulus of 320 Pa. Both the storage and loss moduli for peptide **3** remained steady independent of frequency up to 50 Hz.

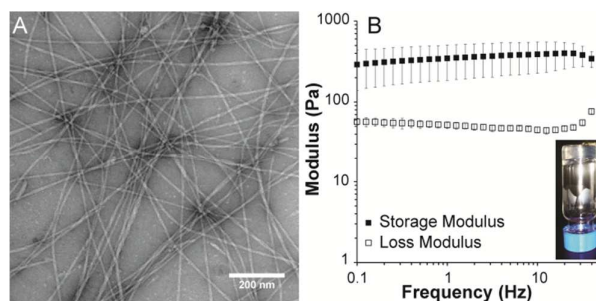


Figure 1. A) Conventional TEM image of peptide **3** cast from water. B) Frequency sweep rheology of peptide **3** at 1 wt. % in water gelled with $CaCl_2$. Inset shows photograph of self-supporting gel.

To evaluate the self-assembled structure of the nanofibers, we first measured the critical micelle concentrations (CMCs) of peptides **2** and **3** using the Nile red assay. Both peptides had CMC values of approximately 1 mg/mL (Figure S6). Next, we characterized peptide **3** by circular dichroism (CD), IR, UV-Vis, and fluorescence spectroscopies. For all four characterization techniques, experiments were performed at a concentration below the CMC (0.25 mg/mL), above the CMC (10 mg/mL), and on the gel (10 mg/mL gelled with $CaCl_2$).

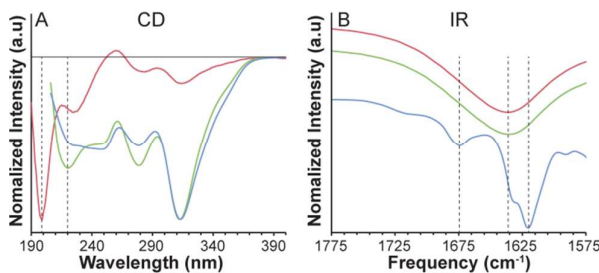


Figure 2. Characterization of peptide **3** at 0.25 mg/mL (red), 10 mg/mL (green) and 10 mg/mL in gel form (blue). A) CD spectroscopy normalized to largest peak. The solid horizontal line represents the 0 line; the vertical dashed lines highlight the peaks indicative of random coil (199 nm) and β -sheet (220 nm) structures. B) IR spectroscopy with y-axes shifted for clarity. The vertical dashed lines designate the amide I absorptions indicative of random coil (1637 cm^{-1}) and β -sheet (1674 and 1620 cm^{-1}) conformations.

Self-assembling peptides often have strong intermolecular hydrogen bonds that align with the long axis of the self-assembled nanofibers, typically taking on a β -sheet confirmation. This type of assembly results in a minimum at 220 nm and maximum at 190 nm in the CD spectrum.¹⁵ Peptide **3** exhibits a minimum at 199 nm, which most resembles a random coil confirmation (Figure 2A). Above the CMC, however, a minimum at 220 nm is observed. Unfortunately, the high concentration (10 mg/mL) limited our ability to reliably collect data below 205 nm, so the expected maximum at 190 nm could not be observed. A similar spectrum is observed upon gelation at 10 mg/mL, indicating that a β -sheet structure likely forms above the CMC and persists in the gel phase. Interestingly, a negative peak at 313 nm increases in intensity upon aggregation and gelation, likely indicating that the SATO chromophore resides in a chiral environment above the CMC of the peptide.

To further investigate the peptide secondary structure, IR spectroscopy was used to analyze the amide I band region (1600–1700 cm^{-1}) of peptide **3**. A strong absorption near 1630 cm^{-1} and a weaker absorption near 1680 cm^{-1} is typically observed when for β -sheets are dominant.¹⁶ In contrast, absorptions for α -helices and random coils are both located near 1650 cm^{-1} . Below the CMC, an absorption peak centered at 1637 cm^{-1} with no secondary absorption near 1680 cm^{-1} was observed for peptide **3**. Above the CMC the absorption shifts to lower frequency at 1635 cm^{-1} , but still without a secondary peak. Upon gelation, however, absorption peaks at 1620 cm^{-1} and 1674 cm^{-1} are present, indicative of a β -sheet structure.

UV-vis and fluorescence spectroscopy data (Figure S7) showed absorptions at around 320 nm and emissions with peaks near 470 nm at all concentrations. No substantial redshift in fluorescence upon aggregation or gelation was observed. Taken together, the spectroscopy data reveal that peptide **3** adopts a random coil conformation below the CMC and increases in β -sheet character above the CMC and upon gelation, and that extensive aromatic stacking is not present in these aggregates but may play a minor role in self-assembly.

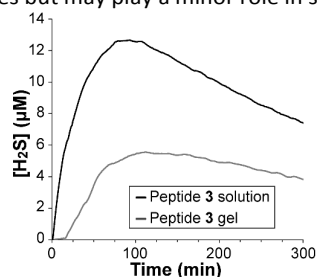


Figure 3. H_2S release from peptide **3** (100 μM) in solution and as an aqueous gel (total of 100 μM in peptide **3**). Both experiments were run in PBS containing 1 mM Cys.

The rate of H_2S release from peptide **3** was measured using an H_2S -selective microelectrode (Figure 3). This method allows for real-time monitoring of the concentration of H_2S in solution. Peptide **3** in solution released H_2S more quickly than in gel form with an earlier peaking time (91 vs. 110 min) and a higher peak concentration (12.6 μM vs. 5.5 μM). Additionally,

a period of slow initial release was observed for the gel, in contrast to the soluble peptide that immediately began releasing H_2S upon addition of Cys. We attribute this induction period and overall slower release in gel form to limited diffusion of Cys into the gel. Importantly, measureable concentrations of H_2S were released from the gel for over 15 h (Figure S8). No H_2S release was observed in the absence of Cys.

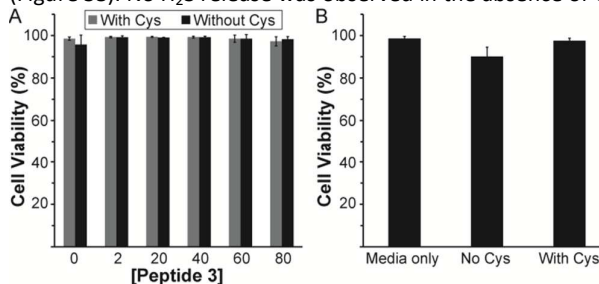


Figure 4. Cytotoxicity of peptide **3** on endothelial cells in basal media with or without Cys (1 mM). A) In solution. B) As 1 wt. % aqueous gel. Viability was measured after 24 h by live/dead staining (calcein AM and ethidium homodimer).

To assess whether peptide **3** could have potential use in vivo, a cytotoxicity study on mouse brain endothelial cells was conducted. Cytotoxicity of peptide **3** in solution was evaluated with and without Cys by measuring cell viability after 24 h (Figure 4A). No toxicity was observed for the peptide **3** solution in either case up to 80 μM . Cytotoxicity of the peptide **3** gel at 1 wt. % was also measured with and without Cys (Figure 4B). Again, no significant toxicity was observed.

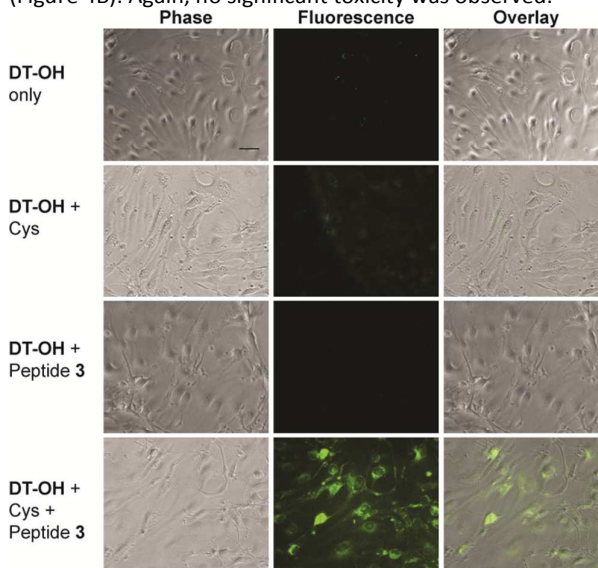


Figure 5. Phase, fluorescence, and overlay Images showing strong fluorescence in endothelial cells preincubated with H_2S probe DT-OH (5 μM) and then treated with peptide **3** (100 μM) and Cys (500 μM). Scale bar = 50 μm .

Lastly, H_2S release from peptide **3** in the presence of endothelial cells was analyzed using a turn-on fluorescent probe selective for H_2S (DT-OH).¹⁷ The addition of peptide **3** and Cys to cells pre-incubated with DT-OH showed strong fluorescence (Figure 5). To confirm the fluorescence was a

result of the H₂S released from the peptide, several controls were performed: **DT-OH** only, **DT-OH** plus Cys, and **DT-OH** plus peptide **3** without Cys. Fluorescence was observed in neither the **DT-OH** only group nor the **DT-OH** + peptide **3** group. Weak signal was observed in the **DT-OH** + Cys group, which we attribute to a previously reported side reaction of similar probes with Cys.^{3b} In future studies we will probe whether H₂S release from peptide **3** occurs in solution followed by diffusion into cells, or whether peptide **3** is internalized into cells before thiol-triggered H₂S release occurs.

Conclusions

In summary, we have designed and synthesized an H₂S-releasing peptide that self-assembles into a robust hydrogel. The self-assembly of these peptides was guided primarily through β -sheet formation with a possible minor contribution from aromatic stacking interactions. Moreover, this peptide demonstrates that the aromatic component of self-assembling aromatic peptide amphiphiles can be designed to be both a structural unit as well as a reactive functional unit that decomposes to release a bioactive small molecule. These results also suggest that such gels may have therapeutic potential for localized H₂S delivery. Investigations are currently underway in our laboratory to further explore the self-assembled structure of these peptides and to evaluate their applications as therapeutic H₂S-releasing materials.

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