Self-assembled nanostructures regulate H$_2$S release from constitutionally isomeric peptides

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Materials
All Fmoc amino acids and Rink amide MBHA resin were purchased from Peak Polypeptide Biosystems LLC (P3BioSystems, Louisville, KY, USA). 4-Formylbenzoic acid (FBA) was supplied by Chem-Impex International, Inc (Wood Dale, IL, USA). O-Benzylhydroxylamine hydrochloride and thiobenzoic acid were provided by Sigma-Aldrich (St. Louis, MO, USA). Doxorubicin hydrochloride (Dox) was supplied by TCI America. All other reagents were sourced from Sigma-Aldrich (St. Louis, MO) or VWR (Radnor, PA, USA), unless otherwise stated. S-Benzoylthiohydroxylamine (SBTHA) was synthesized according to our previous report.

Cell culture
Cell studies were conducted on an adherent H9C2 line of rat embryonic cardiomyocytes (ATCC, Manassas, VA, USA). Cultures were grown in Dulbecco’s Modified Eagle Medium (DMEM, VWR, Radnor, PA), supplemented with 10% fetal bovine serum (FBS, VWR, Radnor, PA), 50 IU/mL penicillin, and 50 μg/mL streptomycin (MP Biomedicals). Cells were cultured at 37 °C in 5% CO$_2$-air. The cultures were passaged after 70–80 % confluence was achieved. Cells were rinsed with 1X PBS solution three times, and then released with trypsin and 0.25% EDTA solution (VWR, Radnor, PA). The suspension of released cells was centrifuged at 1000 rpm for 5 min. The culture protocol for breast cancer cell line MCF-7 (ATCC, Manassas, VA, USA) was the same as for the H9C2 cells.

Peptides synthesis
All peptides used in this study were manually synthesized using standard 9-fluorenylmethoxycarbonyl (Fmoc) solid phase synthesis techniques at a 0.5 mmol scale. Fmoc deprotection steps were carried out by treating the Rink amide MBHA resin with
4-methylpiperidine in dimethylformamide (DMF) (20% v/v). Amino acid coupling steps were performed following Fmoc deprotection by treating the resin with Fmoc-amino acid, O-benzotriazole-\(N,N',N'',N'\)-tetramethyluronium hexafluorophosphate (HBTU), and diisopropylethylamine (DIEA) (4:3.96:10 molar ratio to amine groups on resin) in DMF for 2 h. After coupling of the final amino acid, removal of the Mtt protecting group on derived from Fmoc-Lys(Mtt)-OH was achieved by treating the resin with trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/DCM (3:5:92). FBA was then coupled to the Lys \(\varepsilon\)-amine using the same coupling conditions as noted above for Fmoc amino acids. The final Fmoc group was then removed, and the N-terminus was acetylated by treating the resin with a mixture of acetic anhydride (10 mL, 20% in DMF) and DIEA (80 \(\mu\)L) three times. Finally, peptides were cleaved from the resin by treatment with a TFA/H\(_2\)O (97.5:2.5) solution (15 mL) for 3 h. The liquid was concentrated in vacuo and triturated with cold diethyl ether to precipitate the crude peptide. Following centrifugation to remove the supernatant liquid, the crude materials were dissolved in the mixture of water and acetonitrile containing 0.1% NH\(_4\)OH for purification.

All peptides were purified by preparative RP-HPLC using an Agilent Technologies 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA) equipped with a fraction collector. Separations were performed using an Agilent PLRP-S column (100 Å, 10 \(\mu\)m, 150 \(\times\) 25 mm) monitoring at 220 nm. The expected mass was confirmed using ESI-MS (Advion ExpressIon Compact Mass Spectrometer). Fractions containing pure products were combined and lyophilized (FreeZone \(-105\text{ °C}, \text{Labconco, Kansas City, MO}\)), and then stored at \(-20\text{ °C}\) until needed.

**Synthesis of peptide–H\(_2\)S donor conjugates (PHDCs)**

All PHDCs were synthesized according to previously published methods. An example synthesis is as follows: Lyophilized FBA-terminated peptide (50 mg, 60 \(\mu\)mol) and SBTHA (70 mg, 460 \(\mu\)mol) were dissolved in dry DMSO (700 \(\mu\)L). TFA (10 \(\mu\)L) was added to catalyze the reaction. After standing for 4 h at rt, the solution was diluted with HPLC grade water and acetonitrile (8 mL in total, v:v, 1:1). The pH of the solution was adjusted to 7 by addition of aliquots of 0.1 g/mL aqueous NaOH before purification by preparative RP-HPLC. Product-containing fractions were combined and lyophilized to afford each compound as a white powder. Control peptide K\(_{O}EEK_{O}\) was synthesized in the same way except using \(O\)-benzylhydroxylamine hydrochloride instead of SBTHA (TFA was not included in this step).
Figure S1. Molecular structures of all isomeric PHDCs and the control peptides studied in this manuscript.
Figure S2. ESI mass spectra of \( K_S E K_S E \) (A), \( K_S K_S E E \) (B), \( K_S E E K_S \) (C), \( E K_S K_S E \) (D) and \( K_O E E K_O \) (E).

Characterization of Self-Assembled Peptides

Conventional transmission electron microscopy (TEM): 1 mM stock solutions of PHDCs in 1X phosphate buffered saline (PBS) solution were prepared by direct dissolution of the lyophilized powders. Samples were allowed to age overnight. An aliquot of each solution was removed immediately prior to TEM sample preparation and diluted with 1X PBS to 100 µM. Next, 10 µL of this solution was deposited on a carbon-coated copper grid (Electron Microscopy Services, Hatfield, PA, USA) and allowed to stand for 5 min. Excess solution was wicked away by a small piece of filter paper, and then DI water was deposited for 40 s and wicked away to wash away excess salts. Finally, 10 µL of a 2 wt % aqueous uranyl acetate (UA) solution was deposited on the grid for 5 min. A thin layer was generated after carefully wicking away excess UA. The sample grid was then allowed to dry at rt prior to imaging. Bright-field TEM imaging was performed on a Philips EM420 Transmission Electron Microscope operated at an acceleration voltage of 100 kV. TEM images were recorded by a slow scan CCD camera.

Figure S3. Conventional TEM characterization illustrates the effect of SATO position on the self-assembled morphology of the constitutionally isomeric tetrapeptides. (A) Twisted ribbons formed by \( K_S E K_S E \) in aqueous solution; (B) Twisted ribbons formed by \( K_S K_S E E \) in aqueous solution; (C) Nanocoils formed by \( K_S E E K_S \) in aqueous solution. All grids were stained with UA prior to imaging.

Cryogenic TEM (Cryo-TEM): Cryo-TEM imaging was performed on a FEI Tecnai 12 TWIN transmission electron microscope, operating at 80 kV. Before sample depositing, the TEM grid was treated with plasma air to render the lacey carbon film hydrophilic. 1 mM PHDCs in 1X PBS solution were prepared by direct dissolution of the lyophilized powders and aged overnight. A small drop (3-5 µL) of sample solution was placed on a holey carbon film supported on a TEM copper grid (Electron Microscopy Services, Hatfield, PA). A thin film of the sample solution was produced using a Vitrobot with a controlled humidity chamber (FEI). After loading of the sample solution, the lacey carbon grid was blotted using preset parameters and plunged instantly into a liquid ethane reservoir precooled by liquid nitrogen. The vitrified samples were then transferred to a cryo-holder and cryo-transfer stage that was cooled by liquid nitrogen. To prevent sublimation of vitreous water, the cryo-holder temperature was maintained below -170 °C during
the imaging process. All images were recorded by a 16 bit 2K × 2K FEI Eagle bottom mount camera. The widths and pitches of each nanoassembly was measured by ImageJ and presented as mean ± STD.

**Atomic force microscopy (AFM)** was conducted using a Veeco BioScope II AFM in tapping mode in air at room temperature using Nano World Pointprobe-silicon SPM Sensor tips (spring constant = 1.2-29 N m⁻¹, resonance frequency = 76-263 kHz). The sample for AFM was drop cast from a solution of K₅EEK₅ (100 μM in 1X PBS diluted from 1 mM stock solution) onto freshly cleaved highly-ordered pyrolytic graphite (HOPG).

![AFM height image of K₅EEK₅ on HOPG. Nanocoil heights (highlighted by red rectangle) were 21 ± 3 nm, implying that the nanocoils were rigid enough to preserve their morphology during sample drying.](image)

**Figure S4.** AFM height image of K₅EEK₅ on HOPG. Nanocoil heights (highlighted by red rectangle) were 21 ± 3 nm, implying that the nanocoils were rigid enough to preserve their morphology during sample drying.
**Figure S5.** Representative conventional TEM image of \( \text{EK}_5\text{K}_5\text{E} \), prepared and stained as noted above. It can be seen that \( \text{EK}_5\text{K}_5\text{E} \) forms ill-defined aggregates.

**Figure S6.** Representative conventional TEM image of \( \text{K}_0\text{EEK}_0 \), prepared and stained as noted above. It can be seen that \( \text{K}_0\text{EEK}_0 \) forms ribbon bundles.

**Small angle x-ray scattering (SAXS):** Small angle x-ray scattering pattern of \( \text{K}_5\text{EEK}_5 \) solution was obtained with a SAXSLAB GANESHA 300-XL. CuK\(_\alpha\) radiation was generated by a Genix 3D Cu-source with an integrated monochromator, 3-pinhole collimation and a two-dimensional Pilatus 300K detector. The scattering intensity \( q \) was recorded at intervals of \( 0.012 < q < 0.3 \ \text{Å}^{-1} \) (corresponding to lengths of 10-800 Å). Measurements were performed under vacuum at the ambient temperature. The scattering curve was corrected for counting time and sample absorption. The solution under study was sealed in thin-walled quartz capillaries about 1.5 mm in diameter and 0.01 mm wall thickness. 1 mM \( \text{K}_5\text{EEK}_5 \) solution were prepared by direct dissolution of the lyophilized powder in 1X PBS (pH 7.4) and aged overnight. The scattering spectrum of the solvent was subtracted from the corresponding solution data using the Irena package for analysis of small-angle scattering data. \(^3\)
Model fitting of SAXS data

Quantitative data analysis of $\text{K}_\text{S}\text{EEK}_\text{S}$ was based on fitting its scattering curve to a model of spherical units packed into a helical structure provided by the SASfit software. In this model the scattering intensity was calculated according to:

\[
I(q) = P_{rod}(q, H) \sum_{j=0}^{\infty} \varepsilon_j \left[ \frac{nH}{P} \Psi_j \left( qD, \frac{2\pi j}{Pq} \right) \Phi(q, R) \right]^2
\]

(Eq.S1)

with $\varepsilon_j = 1$ for $j = 0$ and $\varepsilon_j = 2$ for $j \geq 1$

Where

\[
\Psi_j \left( qD, \frac{2\pi j}{Pq} \right) = \begin{cases} 
J_j \left( \frac{qD}{2} \right) & \text{for} \quad q \geq \frac{2\pi|j|}{P} \\
0 & \text{for} \quad q < \frac{2\pi|j|}{P}
\end{cases}
\]

$J_j$ are Bessel functions

$\Phi(q, R)$ is the form factor of a sphere: $\Phi(q, R) = 3 \frac{4\pi}{3} R^3 (\eta_\text{sol} - \eta) \left( \frac{\sin(qR) - qR \cos(qR)}{(qR)^3} \right)$

And $P_{rod}$ is the form factor of infinitesimal thin rod of length $H$

To complement the findings from the cryo-TEM and AFM, we performed small angle X-ray scattering (SAXS) experiment of $\text{K}_\text{S}\text{EEK}_\text{S}$ aqueous solution. As shown in Fig. S7A, the scattering pattern exhibits peaks, characteristic of helical nanostructure and can be fitted to a model of spherical units packed into a helical structure. (The best fit to the model is presented as solid lines in Fig. S7A) yielding the mean diameter of the helical structure, $D=190$ Å, mean radius of the monomer, $R=25$ Å, and the helical pitch of the structure, $P=325$ Å. These parameters are in good agreement with the parameters obtained from the TEM micrographs in Fig. 1F and Fig. S7A.
**Figure S7.** (A) The best fit (solid black line) of the helix model described by Eq.S1 to the SAXS curve of $\text{K}_8\text{EEK}_8$; (B) TEM micrograph of $\text{K}_8\text{EEK}_8$; (C) Schematic illustration of the helix model.

**Fluorescence Spectroscopy:** Nile Red was used as a fluorescent probe to determine critical aggregation concentration (CAC) of each PHDC. Specifically, 10 μL of a 50 μM solution of Nile Red in acetone was added to each vial and dried under vacuum. PHDC in PBS solutions (300 μL) with different concentrations were added to each vial and aged overnight. Fluorescence spectroscopy was performed on a Varian Cary Eclipse fluorescence spectrophotometer with a scanning speed of 200 nm min$^{-1}$, a 1 nm data pitch, an integration time of 0.1 sec, and 10 nm slits. The excitation wavelength was 550 nm, emission was monitored from 570 to 720 nm. The CAC value was taken to be the intersection between the linear fits of the high and low concentration regimes.

**Figure S8.** Fluorescence plots of $\text{K}_8\text{EK}_8\text{E}$ (A), $\text{K}_8\text{K}_8\text{EE}$ (B) and $\text{K}_8\text{EEK}_8$ (C) against concentration in 1X PBS.

**Table S1 CAC values of PHDCs calculated from Figure S8**

| PHDCs   | CAC (μM) |
|---------|----------|
| $\text{K}_8\text{EK}_8\text{E}$ | 35.9     |
| $\text{K}_8\text{K}_8\text{EE}$ | 35.6     |
| $\text{K}_8\text{EEK}_8$    | 18.9     |
Circular dichroism (CD) spectroscopy: The CD spectra of PHDCs from 190 nm to 400 nm were recorded on a Jasco J-815 spectropolarimeter (JASCO, Easton, MD, USA) using a 1 mm path length quartz UV-Vis absorption cell (Thermo Fisher Scientific, Pittsburgh, PA, USA). The 100 μM samples diluted from 1 mM stock solution in phosphate buffer (pH=7.4) were used in these measurements. A background spectrum of the solvent was acquired and subtracted from each sample spectrum. All measurements were conducted in triplicate.

UV-vis spectroscopy: The UV-vis spectra of PHDCs from 200 nm to 400 nm were recorded on a Varian Cary 100 UV-vis spectrophotometer (Agilent Technologies, Santa Clara, CA) using a 1 cm path length quartz UV-Vis absorption cell (Thermo Fisher Scientific, Pittsburgh, PA, USA). The 100 μM samples diluted from 1 mM corresponding stock solutions in 1X PBS (pH=7.4) were used in the measurements. A background spectrum of the solvent was acquired and subtracted from each sample spectrum.

Figure S9. UV-Vis spectra of KsEKS, KsKS EE and KsEEKS in 1X PBS at a concentration of 100 μM. The absorption at around 320 nm is assigned to the SATO functional group.

Calibration of H2S Selective Probe
A diethylenetriaminepentaacetic acid (DTPA) solution was prepared at 150 μM in 1X PBS (pH=7.4). The solution was purged vigorously with nitrogen for 20 min. Anhydrous Na2S was added to a vial in an inert atmosphere glovebox. After removing from the glovebox, PDTA solution (20 mL) was added to make a 5 mM H2S solution. Another scintillation vial was then charged with 20 mL PBS buffer (pH = 7.4) and a small stir bar. The vial was placed on a stir plate, and an H2S-sensitive electrochemical sensor was immersed in the solution, allowing the background current to stabilize for several minutes. Five aliquots of the H2S solution were injected sequentially into the vial (20 μL, 40 μL, 60 μL, 80 μL, 100 μL). 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.

S9
**Hydrogen Sulfide Release Experiments**

The \( \text{H}_2\text{S} \) release experiments were carried out in a special vial shown in the illustration below. An example release experiment at high concentration is as follows: 100 \( \mu \text{L} \) of a stock PHDC solution in PBS was mixed with 10 \( \mu \text{L} \) stock cysteine solution in PBS within the inner well to make the final concentration of PHDC at 1 mM and Cys at 4 mM. The inner well was then covered with a gas-permeable membrane (Breathe-Easier, Diversified Biotech, Dedham, MA) to prevent Cys from interacting with the electrode. Next, degassed PDTA solution prepared for calibration (5 mL) was added to the vial equipped with a stir bar. An \( \text{H}_2\text{S} \) selective microelectrode (World Precision Instruments) was submerged into this solution, and the output current was collected for 4 h. A plot of \( \text{H}_2\text{S} \) concentration vs. time was constructed using the calibration curve. Experiments were repeated three times for each molecule.

![H2S release setup](image)

**Figure S10.** Illustration of \( \text{H}_2\text{S} \) release setup used in this study.

| PHDCs       | Peaking time at 1 mM | Peaking time at 40 \( \mu \text{M} \) |
|-------------|----------------------|--------------------------------------|
| \( \text{K}_\text{S}\text{EK}_\text{S} \text{E} \) | 143 ± 1              | 43 ± 9                               |
| \( \text{K}_\text{S}\text{K}_\text{S} \text{EE} \) | 127 ± 7              | 40 ± 5                               |
| \( \text{K}_\text{S} \text{EEK}_\text{S} \) | 182 ± 4              | 45 ± 13                              |

**Table S2** \( \text{H}_2\text{S} \) release peaking times of PHDCs at different concentration
Figure S11. H₂S release profiles from 1mM of K₈EK₈E, K₈K₈EE and K₈EEK₈ without cysteine.

Figure S12. H₂S release profile from 1 mM of K₈EEK₈ with 4 mM cysteine.

Morphological Transition during H₂S Release from K₈EEK₈
H₂S release experiments were carried out in a 15 mL centrifuge tube. Specifically, 1 mL of stock K₈EEK₈ solution in PBS was mixed with 100 μL stock cysteine solution in PBS within the centrifuge tube to make the final concentration of K₈EEK₈ at 1 mM and Cys at 4 mM. At various time intervals, 100 μL aliquots of this solution were removed and diluted 10-fold immediately before TEM sample preparation. Samples were prepared and imaged by conventional TEM as noted above.

Monitoring of SATO disappearance from K₈EEK₈ during H₂S release
H₂S release experiments were carried out in a 0.1 mm path length quartz UV-Vis absorption cell
Specifically, 20 μL of stock K₅EEK₅ solution in PBS was mixed with 2 μL stock cysteine solution in PBS within the cell to make the final concentration of K₅EEK₅ at 1 mM and Cys at 4 mM. At various time intervals, the UV-vis spectra of PHDC K₅EEK₅ from 250 nm to 400 nm were recorded on a Varian Cary 100 UV-vis spectrophotometer (Agilent Technologies, Santa Clara, CA). A background spectrum of the solvent was acquired and subtracted from each sample spectrum. The data were then plotted as absorbance at the SATO λₘₐₓ (320 nm) versus time (Figure S13).

**Figure S13.** Disappearance of SATO group of K₅EEK₅ during H₂S release as monitored by UV-vis absorption spectroscopy at 320 nm.

From Figure S13, there is an induction period of ~40 min, which is slightly shorter than that of H₂S release profile shown in Figure 2a. The difference is likely a result of two factors: First, SATO groups are consumed once they react with Cys, immediately showing a decrease in absorption at 320 nm. However, for H₂S release, the probe does not show any signal until the SATO groups are converted into H₂S, which requires further reaction steps. Second, there is a membrane in our release setup (Figure S10). The probe responds only after H₂S passes through the membrane and reaches the electrode. Accordingly, the induction period is longer in the H₂S release profile (Figure 2a) than that of the UV absorption experiment, but the trends are the same.

**Cell Viability Assays**

H9C2 cells were plated in a 96-well plate at a density of 5000 cells per well in 200 μL per well. After culturing for 24 h, cells were washed with 1X PBS three times before 185 μL serum-containing DMEM media was added. Then cells were pretreated with following groups for 30 min: 4 mM PHDCs (10 μL) with 32 mM Cys (5 μL); 3.2 mM Cys (5 μL) with PBS (10 μL); 8 mM H₂S donors (GYY4137 or Na₂S, 10 μL) with PBS (5 μL); 4 mM K₀EEK₀ (10 μL) with PBS (5 μL). Next, stock Dox solution in PBS (5 μL) was added to each well to make the
final Dox concentrations ranging from 0 to 10 µM. After incubation for 24 h, cells were washed three times with PBS and then treated with serum-free DMEM media (100 µL) and 10 µL Cell counting kit 8 solution (CCK-8, Dojindo, Rockville, MD). After incubation for another 3 h to allow for development of the CCK8 dye, absorbance was recorded at 450 and 750 nm using a BioTek Synergy Mx plate reader (BioTek, Winooski, VT). Data were graphed using GraphPad InStat, version 3 (GraphPad Software, Inc., San Diego, CA). For data analysis, multiple comparisons were done using one-way ANOVA followed by Student-Newman-Keuls post-hoc tests for multiple pairwise examinations. Changes were identified as significant if $p$ was less than 0.01. Mean values are reported together with the standard error of mean (SEM) representing the combination of 3 different experimental runs. Cell viability was assessed for MCF-7 cells in the same manner as described here for H9C2 cells.

**Figure S14.** Cell viability of H9C2 cardiomyoctyes treated with different H$_2$S releasing donors and controls for 24 h. The final concentration of Cys was 800 µM, K$_0$EEK$_0$ and K$_s$EEK$_s$ was 200 µM. GYY4137 and Na$_2$S concentrations were 400 µM.
Figure S15. (A) Cell viability of MCF-7 cells treated with Cys only or three different PHDCs \( \text{K}_S\text{EK}_S\text{E}, \text{K}_S\text{K}_S\text{EE} \) and \( \text{K}_S\text{EEK}_S \) in the presence of Cys for 24 h. The final concentration of Cys was 800 \( \mu\text{M} \); \( \text{K}_S\text{EK}_S\text{E}, \text{K}_S\text{K}_S\text{EE} \) and \( \text{K}_S\text{EEK}_S \) was 200 \( \mu\text{M} \). (B) Cell viability of MCF-7 cells pretreated with \( \text{K}_S\text{EK}_S\text{E}, \text{K}_S\text{K}_S\text{EE} \) or \( \text{K}_S\text{EEK}_S \) in the presence of Cys for 30 min before exposure to 5 \( \mu\text{M} \) Dox for another 24 h. The final concentration of Cys was 800 \( \mu\text{M} \), \( \text{K}_S\text{EK}_S\text{E}, \text{K}_S\text{K}_S\text{EE} \) and \( \text{K}_S\text{EEK}_S \) was 200 \( \mu\text{M} \).

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