Supplementary Information

Supramolecular control of heme binding and electronic states in multi-heme peptide assemblies

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Materials and Methods

Peptide Design and Visualization

The peptides were designed rationally with the aid of Hyperchem and Pymol. The peptide molecules were built in Hyperchem assuming a parallel β-sheet conformation. A 'sandwich' structure was created consisting of 2 pentamers (five peptides of PA-$(LK)_3$ or PA-$(KL)_3$) in an idealized β-sheet arrangement (~ 5 Å) with the hydrophobic leucine side facing the interior of the sandwich structure. A decamer of PA-L_3K_3 was created as an idealized β-sheet but no sandwich structure was created due to the block patterining of the leucine and lysine residues as opposed to the hydrophilic/hydrophobic patterning of the leucine and lysine residues in PA-$(LK)_3$ and PA-$(KL)_3$. The structures were partially minimized using the CHARMM27 force field to achieve idealized β-sheet arrays. Heme molecules were also built in hyperchem and incorporated into the structures by forcing an axial ligation to the ε-nitrogen of the histidine residue. This structure was partially minimized using the CHARMM27 force field. The resulting structures were imported into PYMOL and modified for visualization.

Peptide Synthesis, purification and characterization

The synthetic procedure for c16-AHL_3K_3-CO_2H has been reported in our previous studies. The synthesis of c16-AHLKLKLK-$CONH_2$, and c16-AHKLKLKL-$CONH_2$, cleavage from the resin and RP-HPLC purification followed the same strategy as the previously reported peptide with the exception of using rink amide – MBHA resin in place of Wang resin. The first amino acid was double coupled to the RA-MBHA resin to ensure an efficient reaction. MALDI-TOF MS (Bruker UltrafleXtreme MALDI-TOF) was used to identify the peptides; c16-AHL_3K_3-$CONH_2$: Calc'd for $C_{61}H_{114}N_{14}O_9$+ $[H^+]$, 1187.9, found 1188.117 m/z; c16-$AH(LK)_3$-$CONH_2$: Calc'd for $C_{61}H_{114}N_{14}O_9$ + $[H^+]$, 1187.9, found 1188.238 m/z, and c16-$AH(LK)_3$-$CONH_2$: Calc'd for $C_{61}H_{114}N_{14}O_9$ + $[H^+]$, 1187.9, found 1188.457 m/z.

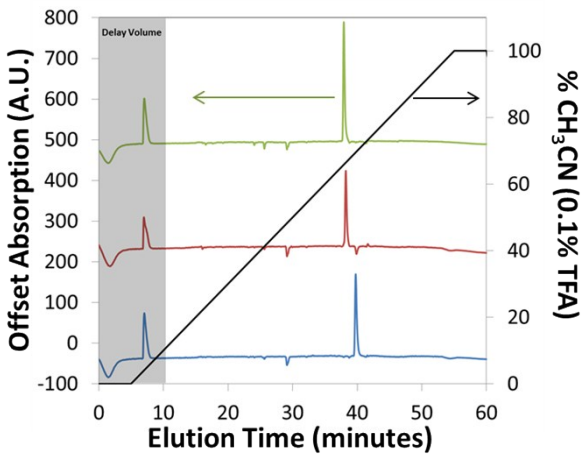

Figure S1. Reversed Phase HPLC chromatograms of PA-L_3K_3 (blue), PA-$(LK)_3$ (red), and PA-$(KL)_3$ (green). 100 μM peptide was dissolved in the mobile phase, water (0.1% trifluoracetic acid). After injection, a gradient of 2% acetonitrile (0.1% trifluoracetic acid) per minute over 50 minutes was employed. >98% purity was achieved. The signals in the delay volume are systematic responses from running consecutive samples (negative peak) and injection (positive peak at ~7 min).

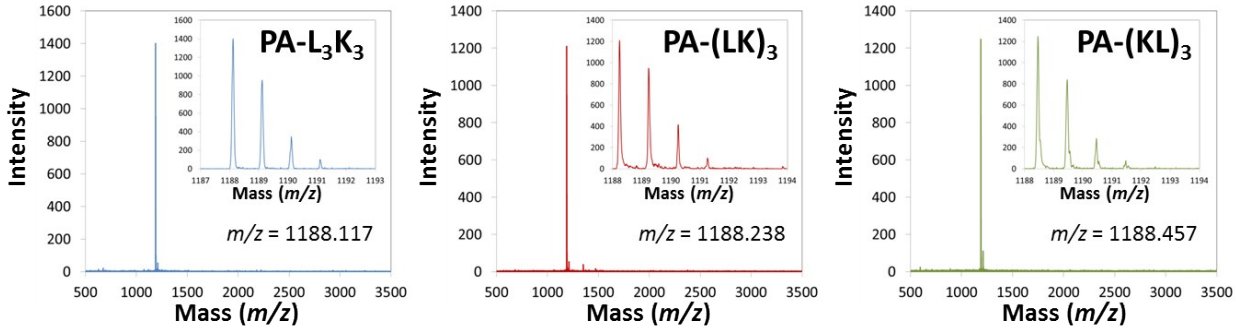

Figure S2. MALDI-TOF Mass Spectrometry of the peptide series. 2', 6' Dihydroxyacetophenone (1 wt%) in 50:50 Water:Acetonitrile (0.1% TFA) was used as the matrix.

Stock Solution Preparation

All stock solutions were used the day of preparation. Each peptide, c16 –AHL_3K_3-CO_2H, c16-$AH(LK)_3$-$CONH_2$, and c16-$AH(KL)_3$-$CONH_2$ (3-4 mg) was dissolved in nanopure water (Millipore A10) to obtain a 1 wt%, 8.4 mM solution. Hemin (Porcine, Sigma-Aldrich) was dissolved in DMSO (Sigma Aldrich) to achieve a 10 mM stock solution. Note: Hemin/DMSO stock solutions were always made to ensure that the final DMSO concentration in the sample was less than 1% (v/v).

Peptide Secondary Structure Characterization

In order to analyze secondary structural formation in the absence of heme, circular dichroism spectroscopy (Jasco, Inc. J-815) was employed to analyze the typical n-π* transitions found for a β-sheet assembly. Assembled stock solutions were prepared by adding 54 μL of the peptide stock into 246 μL of 50 mM HEPES buffer, 100 mM NaCl, pH 7.0 or 20 mM NH_4OH, pH, 10.5 solutions to yield a 1.5 mM stock solution. The samples were heated to 70 ^oC and then cooled. These stock solutions were then diluted into water, 10 μL into 290 μL to afford a 50 μM solution. The samples were transferred to a 1 mm quartz cuvette cuvette (Starna Cells, Inc.) and analyzed by scanning from 260 – 190 nm.

Additional secondary structural characterization was achieved with infrared spectroscopy (Thermo Scientific, Nicolet 6700 FT-IR spectrophotometer). 10 μL of the 1.5 mM samples described in the previous section were dropcast onto a 32 mm CaF_2 plate (Sigma Aldrich) and were air dried. The thin films were aligned in the spectrophotometer and the amide I vibrations in the region from 1500 – 1800 cm^{-1} were analyzed.

Heme Coordination Characterization

Heme was introduced to the 1.5 mM peptide solutions described in the previous section by adding 3 μL of the 10 mM heme stock solution into the 300 μL peptide solution to yield 1.5 mM peptide, 100 μM heme or 15:1 - peptide:heme samples. The samples were then heated to 70 ^oC and cooled to room temperature.

For UV/visible spectroscopic analysis, the samples were transferred to a 1 mm quartz cuvette (Starna Cells, Inc.) and analyzed from 300 – 800 nm. Due to the size of some of the assemblies we observe some light scattering as evidenced by an increase in the baseline absorption. As a result, we correct for the sample scattering by subtracting the absorption value at 800 nm. These same samples were analyzed by CD spectroscopy by scanning the Soret region from 350 – 500 nm. The secondary structure of the heme coordinated assemblies were analyzed by IR spectroscopy only because the addition of DMSO UV cutoff precludes measurement in the 190 – 260 nm range required for CD measurements. Samples for IR were prepared in the same fashion described earlier when heme is no coordinated to the assembly.

X-band continuous wave EPR experiments were carried out using a Bruker ELEXSYS E580 spectrometer operating in the X-band (9.4 GHz) and equipped with an Oxford CF935 helium flow cryostat with an ITC-5025 temperature controller. Samples for EPR (same preparation as described in the 'Sample Preparation' section) were concentrated to 1 mM hemin and 15 mM Peptide with a 10,000 molecular

weight cutoff spin diafiltration system (EMD Millipore Inc., Amicon Ultra-0.5 Centrifugal Filter Unit with Ultracel-10 membrane). All experiments were performed at 10 K with modulation amplitude set to 10 and power set to 10 mW.

Electrochemistry measurements were made with a BASI Electrochemical Workstation. Samples (10 mL of a 1.5 mM peptide 100 μM heme solution) were dropcast and dried on a freshly polished glassy carbon working electrode. A platinum wire served as the auxiliary electrode while a Ag/AgCl reference electrode was used. The measurements were made in 50 mM phosphate buffer, 150 mM NaCl, at pH 7.5. All numbers reported in the text are vs. Ag/AgCl.

Supramolecular Morphology Characterization

Atomic force microscopy (AFM) images were obtained with a Veeco MultiMode 8 scanning probe microscope equipped with a silicon nitride tips (Bruker, SCANASYST Air) for imaging soft-materials. The images were obtained using the instruments peak force mode. The sample was prepared by drop casting 100 μL of a 15 μM (peptide) sample (diluted from a 150 μM solution with or without 10 μM heme) on freshly cleaved mica (Ted Pella) and allowed to incubate for 5 minutes. The excess sample was wicked away with filter paper and washed 3 x with water. The sample was air dried prior to measurements.

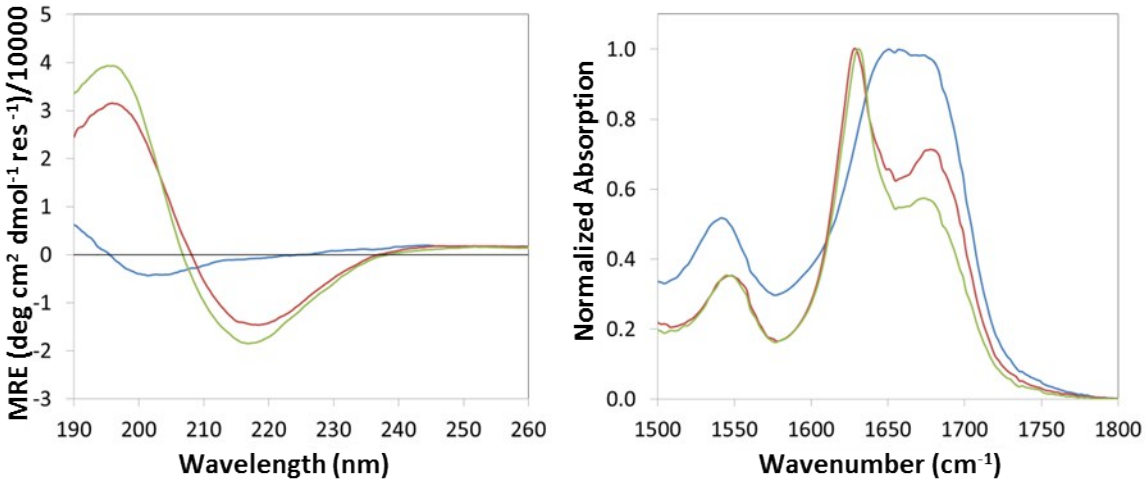

Figure S3. Characterization of the secondary structure of the peptide assemblies without heme in 50 mM HEPES Buffer, 100 mM NaCl, pH 7.0 by (A) CD and (B) FTIR spectroscopies. PA-L_3K_3 (Blue), PA-$(LK)_3$ (red), and PA-$(KL)_3$ (green).

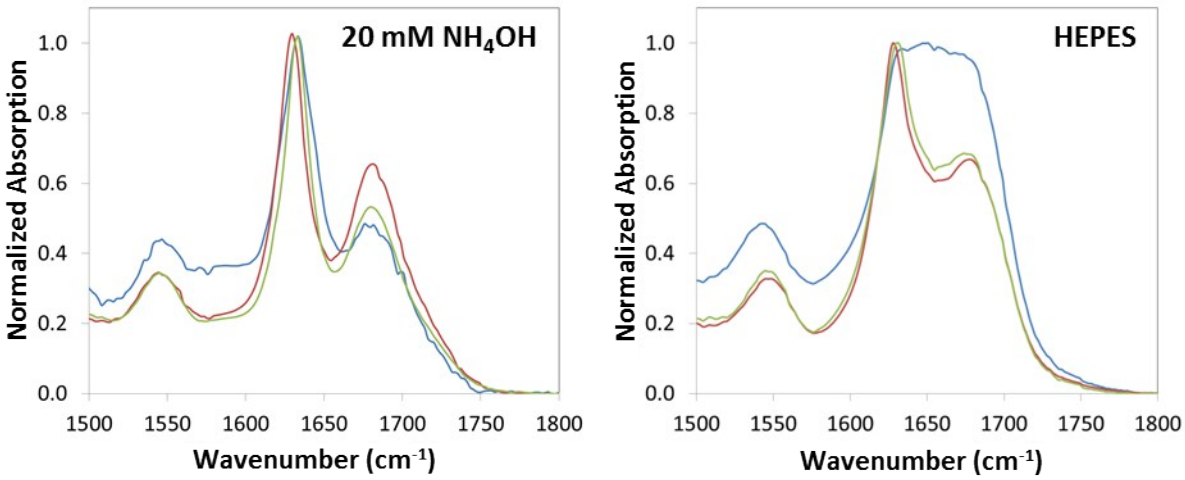

Figure S4. Characterization of the secondary structure of the peptide assemblies **with heme** in (A) 20 mM NH_4OH and (B) 50 mM HEPES Buffer, 100 mM NaCl, pH 7.0 by FTIR spectroscopy. PA-L_3K_3 (Blue), PA-$(LK)_3$ (red), and PA-$(KL)_3$ (green). Samples (10 μL) of 15:1 peptide:heme (1.5 mM peptide, 100 μM heme) were dropcast and air dried on a transparent CaF_2 window.

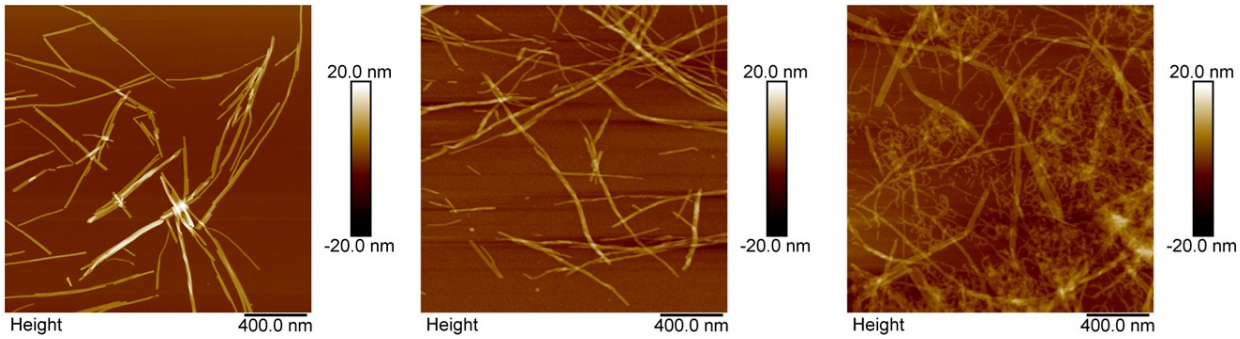

Figure S5. Atomic force micrographs characterizing the supramolecular structure of (A) PA-L_3K_3 (B) PA-$(LK)_3$ and (C) PA-$(KL)_3$ in the absence of heme. The samples were dropcast onto freshly cleaved mica from a solution of 15 μM peptide sample in 20 mM NH_4OH.

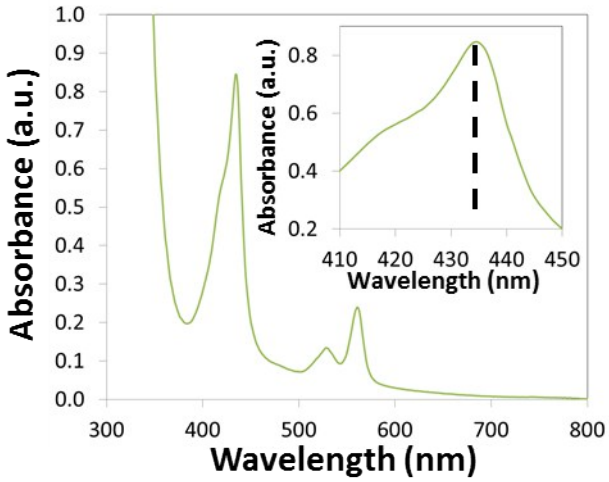

Figure S6. UV/visible spectrum of reduced hemin coordinated to PA-$(KL)_3$ in 20 mM NH_4OH. The inset is a detailed close-up to confirm that the reduced, ferrous heme yields a 435 nm Soret band that is distinct from the oxidized, ferric state and that the split Soret is maintained.

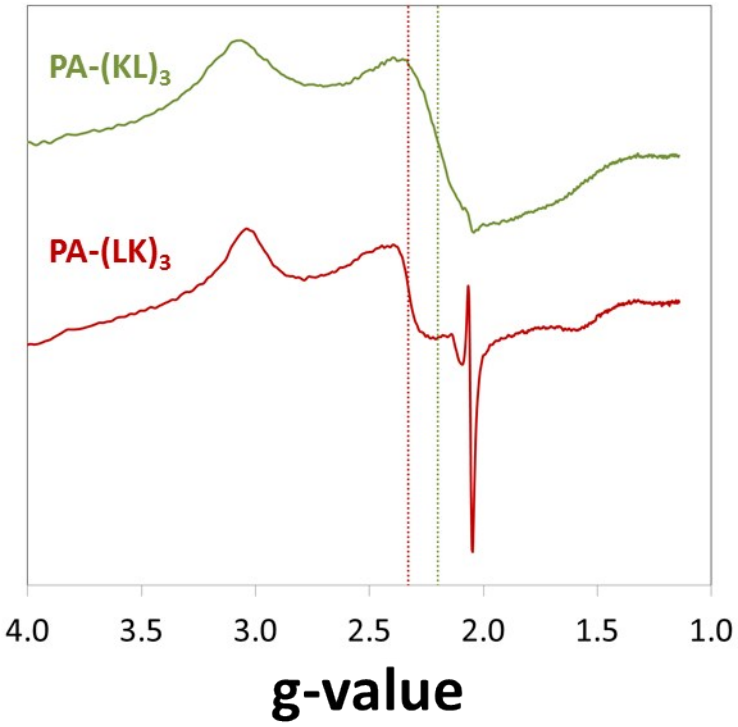

Figure S7. A magnified view of figure 4B in which the low spin characteristics of **PA-$(KL)_3$** and **PA-$(LK)_3$** are highlighted. The vertical lines highlight the difference in the g_y values, 2.33 for PA-$(LK)_3$ (red dashed line) and 2.20 for PA-$(KL)_3$ (green dashed line). The broadening due to spin-spin interactions between neighbouring heme molecules in PA-$(KL)_3$ is highlighted here.

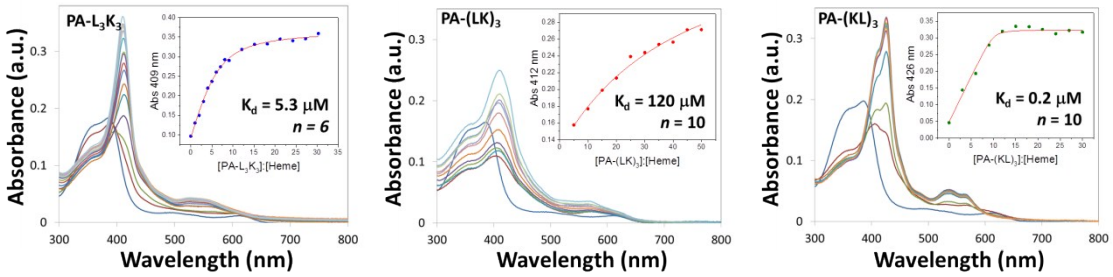

Figure S8. UV/visible monitored titrations of (A) PA-L_3K_3, (B) PA-$(LK)_3$ and (C) PA-$(KL)_3$ where the heme concentration was held at 33 μM and the peptide was varied from 0 to 1 mM in 100 μM increments. The peptide:heme ratio was determined by allowing the ratio 'n' to float, equation S1. We then fixed the ratio to the closest integer: *n* = 6 for PA-L_3K_3 and *n* = 10 for PA-$(KL)_3$ to obtain the final K_d values. PA-$(LK)_3$ lacked a clear inflection point and was assumed to have a 10:1 peptide:heme stoichiometry in order to quantitatively compare the binding affinity. The calculated binding constant curves are represented as solid red lines.

A = Ao + ((ϵ_B * l * 0.5) * ((x * M/n + Kd + M) - ((x * M/n + Kd + M)^2 - (4 * x/n * M^2))^0.5)) - (ϵ_s * l * M) + ((ϵ_s * l * 0.5) * (x * M/n + Kd + M) - (((x * M/n + Kd + M))^2 - (4 * x/n * M^2))^0.5)

Same as:

$$A = Ao + \frac{\epsilon_B l}{2} \times \left(\left(\frac{x}{n} \times M + K_D + M\right) - \sqrt{\left(\frac{x}{n} \times M + K_D + M\right)^2 - 4 \times \frac{x}{n} \times M^2}\right) - \epsilon_S \times l$$

Equation S1. The equation employed for stoichiometry and binding constant analysis. The top equation can be cut and pasted into origin functions. The bottom equation represents the same equation in a more readable format. Ao, initial absorption; ϵ_B, extinction of bound heme; ϵ_s, extinction coefficient contribution from scattering; x, ratio of peptide to heme; n, set stoichiometry of peptide to heme; K_d, dissociation (binding) constant M, molar concentration of heme; l, cuvette pathlength.

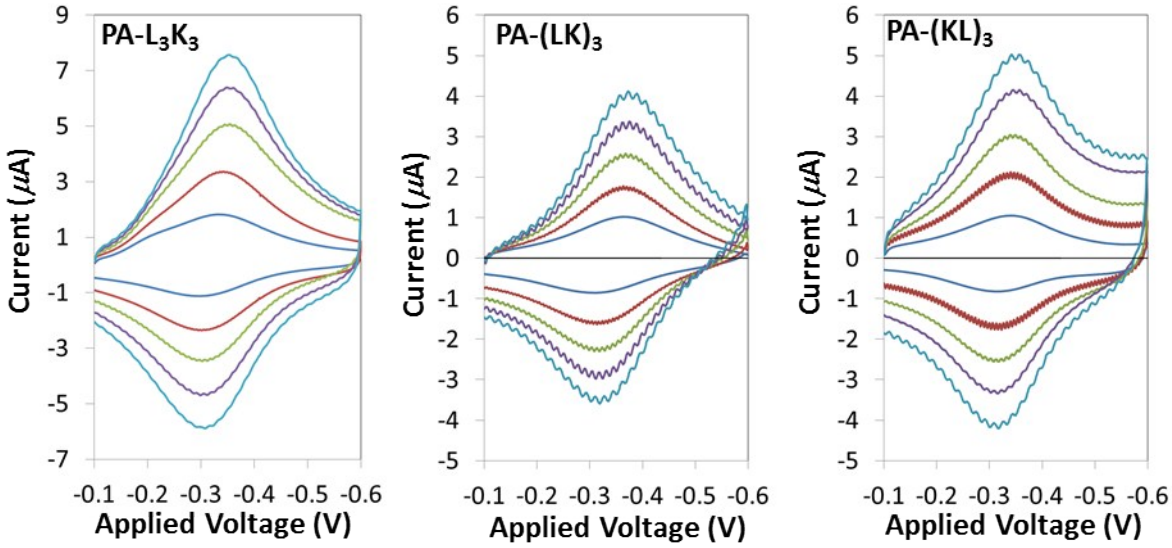

Figure S9. Thin film voltammetry results at various scan rates: 200, 400, 600, 800, and 1000 mV/s for (A) PA-L_3K_3 (B) PA-$(LK)_3$ and (C) PA-$(KL)_3$. The applied voltage is vs. Ag/AgCl reference electrode.