Supporting information

Racemic peptide assembly boosts biocatalysis

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S1. Materials and Methods

All reagents and chemicals were purchased from Merck (Milan, Italy) without further purification, except for the Rink amide linker and the 2-chlorotrityl chloride resin that were obtained form Iris Biotech GmbH (Marktredwitz, Germany). An in-line Millipore RiOs/Origin system provided MilliQ water with a resistivity higher than 18 M Ω cm. LC-MS analyses were performed on an Agilent 6120 single-quadrupole system. was obtained from. ^{1}H-NMR and ^{13}C-NMR spectra were recorded at 400 MHz and 100 MHz, respectively, using a 400 MHz Varian Innova Instrument, with chemical shift reported as ppm.

S2. Solid-phase peptide synthesis

L-His-D-Phe-D-Phe-$CONH_2$ and L-His-D-Phe-D-Phe-$CONH_2$ were prepared by solid-phase peptide synthesis using a Rink amide linker on a 2-chlorotrityl chloride resin, with standard protocols for Fmoc-protection strategy and Oxyma Pure B as coupling agent. The crudes were concentrated under argon flow and then dissolved in a mixture of acetonitrile (MeCN, 20%) and milliQ water (80%) with 0.05% trifluoroacetic acid (TFA) that were the solvents used also for HPLC purification. Samples were centrifuged at 15,000 rpm for 10 min., then 0.2 μm filtered prior to injection into the HPLC. Each peptide was purified on an Agilent 1260 with a C-18 column (Kinetex, 5 μm, 100 Å, 250 x 10 mm, Phenomenex), T = 35 °C, flow = 3 mL/min with the following method: t = 0-2 min, 20% MeCN (0.05% TFA); t = 8.5 min, 33% MeCN (0.05% TFA), t = 11-13 min., 95% MeCN (0.05% TFA). R_t = 6.6 min.

S3. Spectroscopic data for D-His-L-Phe-L-Phe-NH_2

The spectroscopic data for this tripeptide corresponded to the literature, as published in M. Kurbasic, Ana M. Garcia, S. Viada, and S. Marchesan, *Molecules* **2020**, *26*, 173: ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.89 (s, 1H, NH), 8.68 (d, J = 8.7 Hz, 1H, NH), 8.47 (d, J = 8.2 Hz, 1H, NH), 8.15 (s, 3H, NH_3^+), 7.44 (s, 1H, Ar), 7.32 – 7.03 (m, 10H, Ar), 6.89 (s, 1H, Ar), 4.76 – 4.64 (m, 1H, αCH), 4.46 (td, J = 8.7, 5.1 Hz, 1H, αCH), 4.15 – 4.01 (m, 1H, αCH), 3.06 (dd, J = 3.9 Hz, J_{gem} = 13.8 Hz, 1H, βCH_2), 3.01 (dd, J = 5.1 Hz, J_{gem} = 13.8 Hz, 1H, βCH_2), 2.90 (dd, J

= 4.7 Hz, J_{gem} = 15.8 Hz, 1H, βCH$_2$), 2.82 (dd, J = 9.2 Hz, J_{gem} = 13.9 Hz, 1H, βCH$_2$), 2.72-2.63 (m, 2H, βCH$_2$). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 173.1, 170.9, 167.3 (3 x CO); 138.3, 137.7, 134.8, 129.7, 129.6, 128.6, 128.4, 126.8, 119.0, 117.3, 115.9 (Ar); 54.6, 54.1, 51.6 (3 x αC); 38.7, 38.2, 26.8 (3 x βC). ESI-MS m/z 449.2 (M+H)$^+$ $C_{24}H_{28}N_6O_3$ requires 449.2.

S4. Spectroscopic data for L-His-D-Phe-D-Phe-NH$_2$

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.79 (s, 1H, NH), 8.69 (d, J = 8.9 Hz, 1H), 8.48 (d, J = 8.4 Hz, 1H), 8.15 (s, 2H, NH$_2$), 7.46-6.88 (m, 14H, Ar, CONH$_2$), 4.70 (td, J = 10.1, 4.0 Hz, 1H, αCH), 4.47 (td, J = 8.9, 5.2 Hz, 1H, αCH), 4.07 (dd, J = 9.2, 3.5 Hz, 1H, αCH), 3.10 – 3.00 (m, 2H, βCH$_2$), 2.92 – 2.80 (dd, J = 13.9, 9.2 Hz, 2H, βCH$_2$), 2.72 – 2.63 (m, 2H, βCH$_2$). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 173.1, 170.9, 167.3 (3 x CO); 138.3, 137.7, 134.8, 129.7, 129.6, 128.6, 128.4, 126.8, 119.0, 117.3, 115.9 (Ar); 54.6, 54.1, 51.6 (3 x αC); 38.7, 38.2, 26.8 (3 x βC). ESI-MS m/z 448.6 (M+H)$^+$ $C_{24}H_{28}N_6O_3$ requires 449.2.

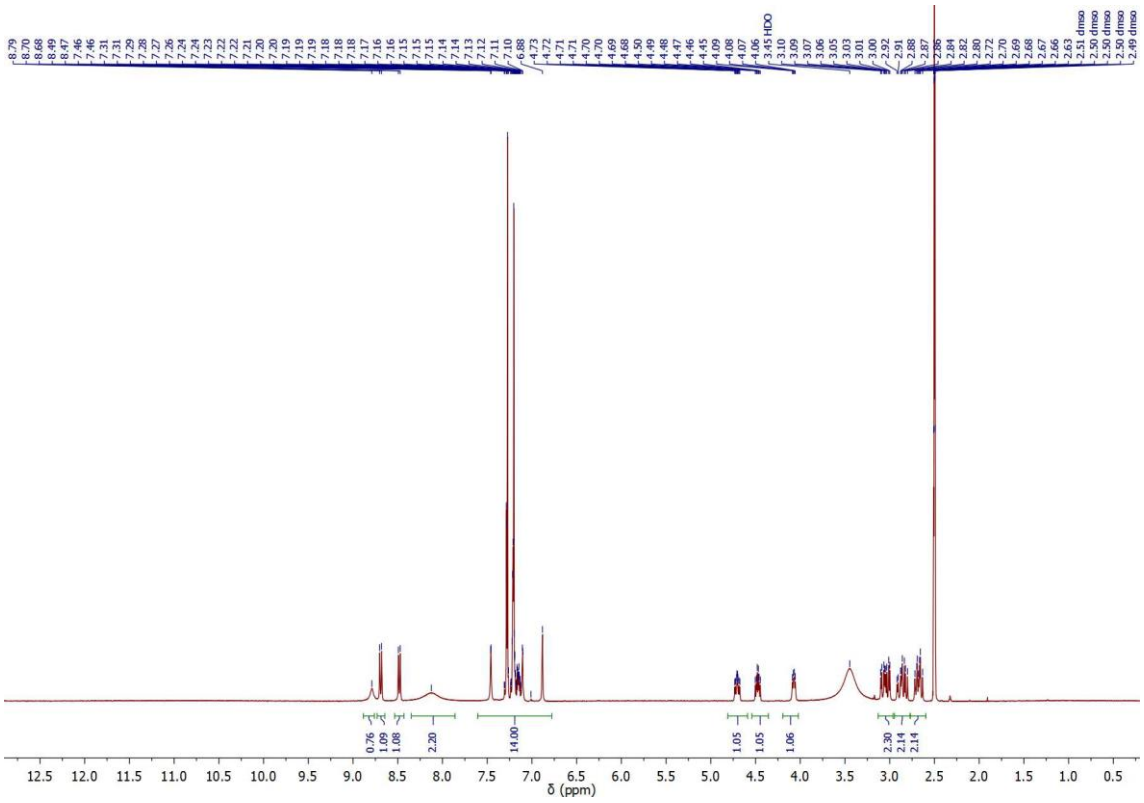

Figure S1. ^{1}H-NMR spectrum of L-His-D-Phe-D-Phe-NH$_2$ (Hff).

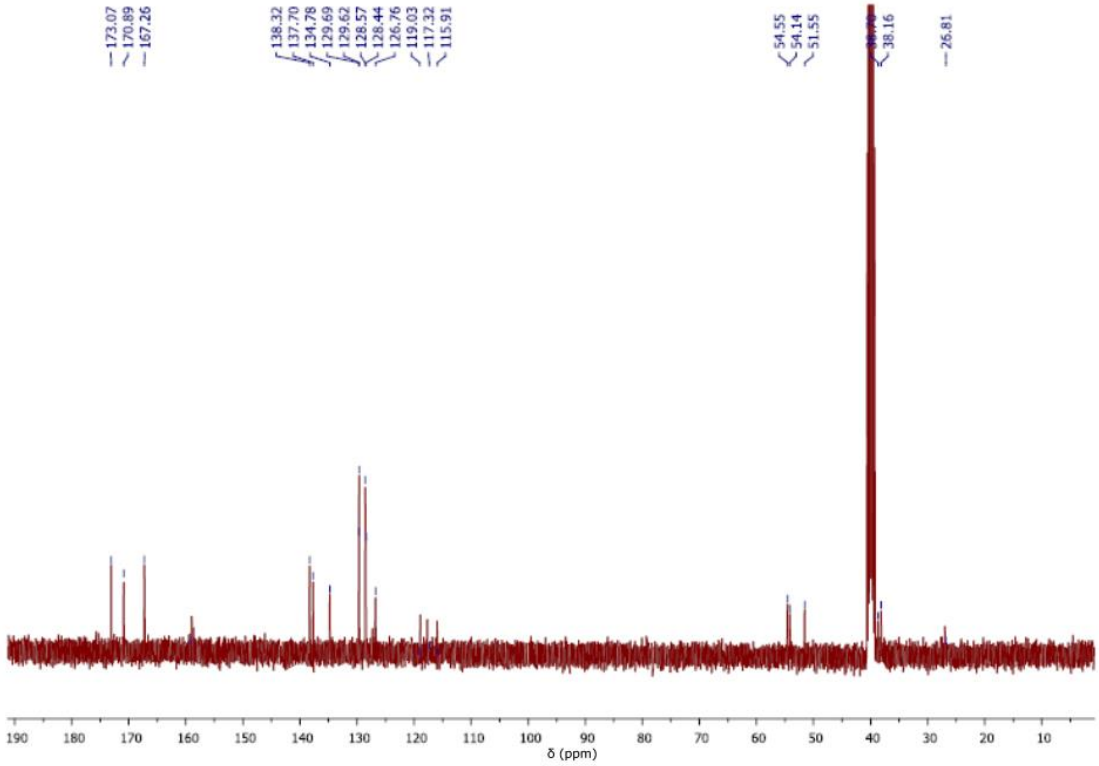

Figure S2. ^{13}C-NMR spectrum of L-His-D-Phe-D-Phe-NH_2 (Hff).

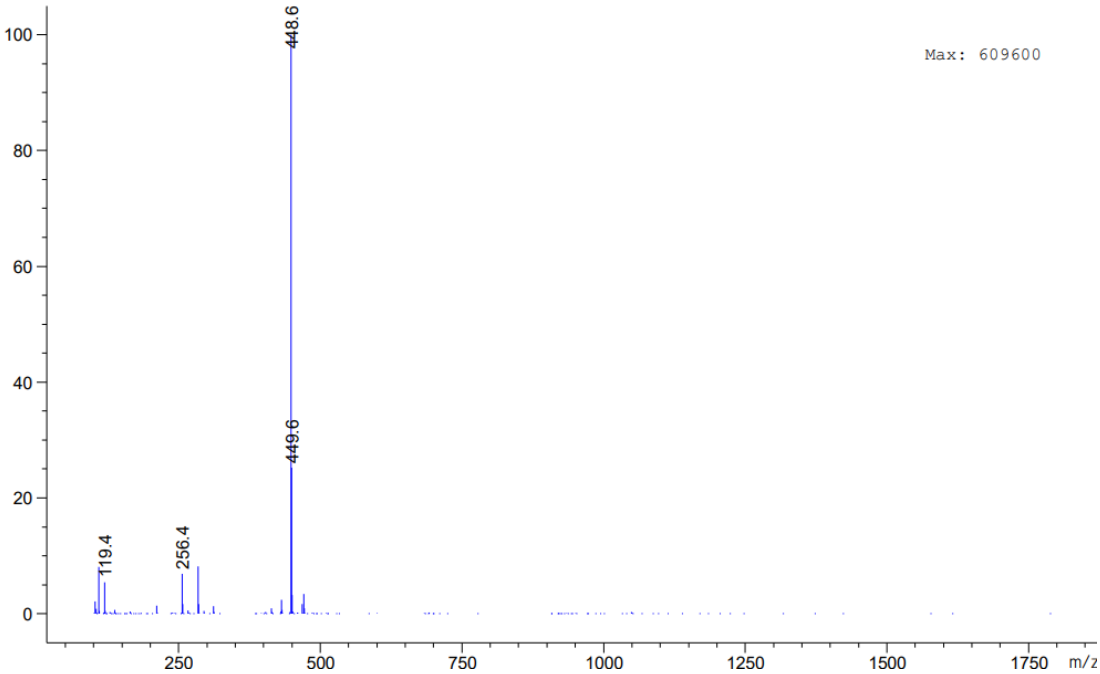

Figure S3. ESI-MS spectrum (positive ion mode) of L-His-D-Phe-D-Phe-NH_2 (Hff).

S5. Circular Dichroism (CD)

CD spectra were recorded using a Jasco J-815 spectropolarimeter with a Xenon lamp of 150 W. The tripeptide solutions (1 mM or 10 mM) were dissolved in alkaline sodium phosphate buffer (0.1M, pH 9.73) and samples were measured in a 0.1 mm quartz cell (Hellma 0.1 mm quartz Suprasils). Spectra were obtained from 190 to 320 nm with a 1 nm step and 0.5 s integration time per step at 20 ºC.

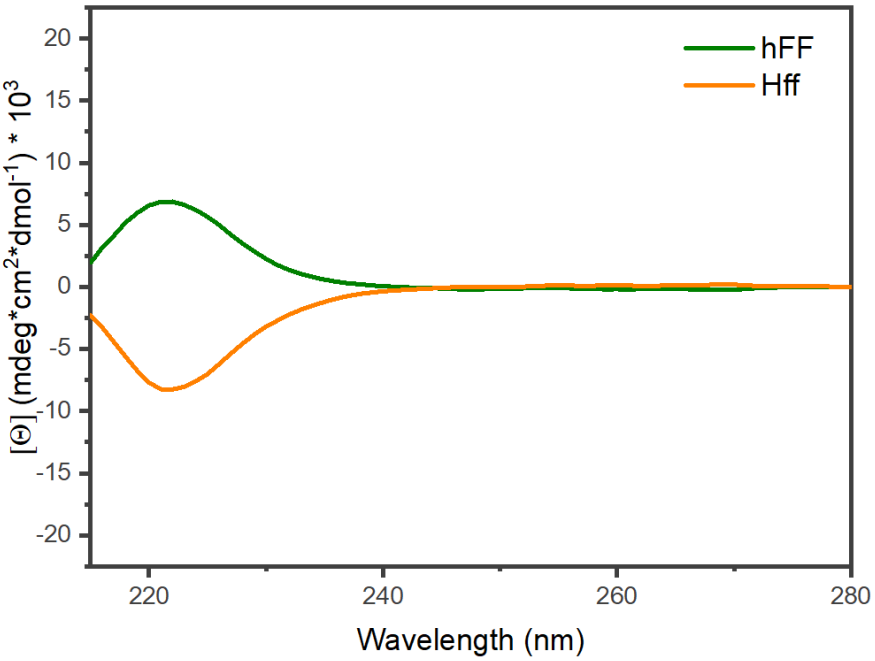

Figure S4. CD spectra of hFF and Hff at 10 mM.

S6. Hydrogelation

Each tripeptide was dissolved to a final concentration of 50 mM in alkaline sodium phosphate buffer (0.1M, pH 9.73). Alternatively, the mixture of both enantiomers was dissolved in a 1:1 ratio, to the overall concentration of 50 mM. To achieve complete dissolution of the peptide(s), samples were sonicated in an HSt Powersonic 603 ultrasonic bath at 37 ºC. Hydrogelation was achieved at a pH of 7.4 ± 0.1. The pH was measured using a HACH sension PH 3 pH meter. The pH meter was calibrated using pH 4, pH 7 and pH 10 buffer solutions.

S7. Fourier transformed infra-red (FTIR) spectroscopy

Spectra were recorded using a Perkin-Elmer Two FTIR ATR spectrometer. The hydrogels were compressed onto the diamond crystal. All spectra were scanned over the range between 2000 and 400 cm^{-1} at a resolution of 4 cm^{-1}. Amide regions are shown.

S8. Atomic Force Microscope (AFM)

The measurements were obtained with a Park NX20 atomic force microscope (Park Systems) operating at 25 ºC and atmospheric pressure. The data resulting from each study was processed using the XEI program (Park Systems).

S9. Scanning Electron Microscopy (SEM)

Hydrogel images were obtained using a FEI Quanta 400 SEM. The hydrogels were subjected to critical point drying with CO_2 and coated with Au-Pd. Images were analyzed with the free software FIJI and 100 counts per sample were used to calculate median fiber diameter and standard deviation. Representative measures are shown below:

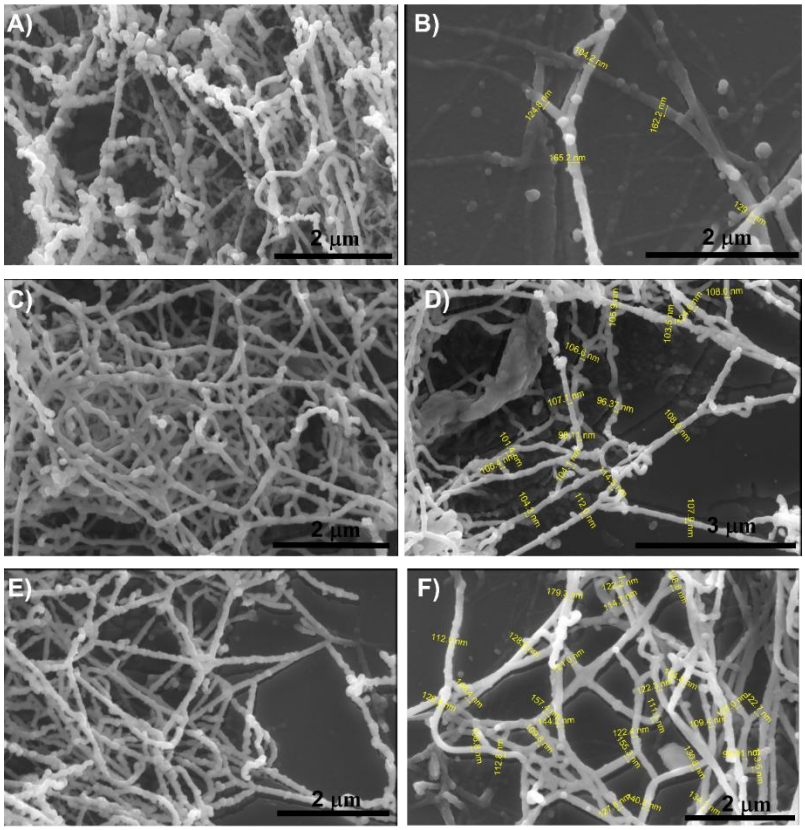

Figure S5. SEM images of tripeptide self-assembly into hydrogels at 50 mM. (A) Image of hFF hydrogel and (B) representative sizes of hFF fibrils. (C) Image of the hydrogel

formed by Hff and (D) representative sizes of Hff fibrils. (E) Image of the hydrogel formed by the racemic mixture and (F) representative sizes of racemic fibrils.

Table S1. Fibrils' diameter for hFF, Hff, and their racemic mixture calculated with FIJI software.

hFF	Hff	Rac.
104.2	106.6	116.6
129.1	105.9	104.2
124.8	103.5	105.7
162.2	124.6	92.6
165.2	108.0	111.6
109.2	106.4	112.4
97.7	101.4	128.6
100.0	104.3	109.6
118.5	104.1	103.8
117.4	112.0	102.6
86.5	114.5	120.5
114.2	98.1	101.8
91.3	96.4	96.1
101.4	107.1	118.9
116.7	106.6	110.6
118.8	108.0	91.9
128.3	107.9	101.6
129.1	165.2	93.9
111.0	113.0	122.5
106.5	118.0	96.5
103.2	87.7	156.0
81.4	105.0	108.0
126.6	91.2	96.9
129.3	88.2	103.2

102.3	90.2	127.0
114.0	106.4	104.2
101.0	97.6	87.4
101.2	90.8	78.2
136.8	101.3	87.7
107.8	102.0	128.9
99.3	103.3	106.2
107.4	101.9	99.0
123.6	95.9	87.3
103.8	104.5	98.5
107.5	86.7	77.3
103.2	107.5	94.1
152.5	84.7	134.6
112.9	96.3	75.3
99.6	89.2	79.8
91.2	87.0	92.7
95.4	97.9	101.5
111.8	87.9	101.5
104.8	100.1	115.0
98.5	96.3	108.9
90.6	86.3	104.5
99.9	100.1	97.4
111.3	101.7	112.4
106.2	109.1	89.9
103.4	119.6	95.0
92.4	117.2	86.8
91.6	117.2	100.5
106.4	100.5	72.7
112.7	97.0	107.6

80.8	98.8	110.3
97.7	100.1	90.8
130.7	87.6	101.5
111.8	92.0	112.0
122.8	132.7	128.8
104.8	76.5	146.4
115.6	103.9	106.8
147.6	120.0	112.8
105.0	104.6	109.8
102.3	108.3	144.2
111.8	98.8	128.6
117.1	111.0	121.0
99.3	89.8	179.3
92.0	107.4	122.2
103.6	99.2	114.7
110.9	97.3	128.8
130.7	111.9	122.3
141.2	141.9	134.8
110.9	117.3	111.1
114.7	110.9	122.4
79.6	107.9	127.8
97.0	110.9	140.8
98.3	106.0	130.8
122.3	105.2	109.4
145.9	97.8	125.0
92.5	84.7	96.9
107.2	88.2	113.5
108.9	120.0	122.7
95.2	101.1	73.5

92.5	94.2	96.9
83.1	83.9	90.8
90.7	79.4	92.5
113.6	88.1	82.9
97.6	85.9	106.8
100.6	97.9	88.8
92.3	172.3	95.0
93.3	121.4	97.0
97.6	126.6	126.3
69.3	136.5	106.8
74.1	91.6	105.9
108.1	130.9	107.7
131.3	168.7	124.1
72.8	121.4	113.1
96.2	99.2	109.3
114.6	123.6	107.6
58.4	141.9	115.1
77.1	127.3	124.4

S10. Oscillatory rheology

Hydrogels were formed *in situ* in an oscillatory rheometer Kinexus Ultra Plus (Malvern, Alfatest, Milan, Italy) using a stainless-steel 20 mm parallel plate geometry at 25 °C. Time sweeps were recorded at a frequency of 1 Hz and a stress of 5 Pa. Stress sweeps were recorded at 1 Hz. Measurements were repeated twice on independent experiments and a representative dataset is shown.

S11. Catalysis

To ensure the formation of homogeneous hydrogels within the 96-well plates and to enable correct absorbance measurements, the gelation protocol was adapted as follows. Each tripeptide was dissolved at 50 mM (when tested alone, or 25 mM each for the

racemic mixture) in 0.1M sodium phosphate pH 11.8 and 73 µl were immediately placed in clear polystyrene Greiner 96 U Bottom wells. Next, 73 µl of 0.1 M sodium phosphate pH 5.6 were added on top to reach a final pH 7.1 ± 0.1 to enable comparison with literature data. Self-assembly was allowed to occur at room temperature for 1 hour. Then, 4 µl of a *p*-nitrophenyl acetate (pNPA) solution (37.5 mM in MeOH) were added to the wells. The absorbance at 405 nm was monitored for on a Tecan Infinite M1000 Pro. Each condition was repeated 4 times. Average absorbance values of blanks (without pNPA) and controls (without peptides) were subtracted from all samples. Higher peptide concentrations were not tested because the racemic gel was too stiff to enable homogeneous diffusion of pNPA.

S12. ANS fluorescence

Each tripeptide was dissolved at 50 mM (when tested alone, or 25 mM each when tested together in the racemic mixture) in 0.1M sodium phosphate pH 11.8, and samples of 75 µl each were transferred onto the well of a black polystyrene 96-well Greiner plates, flat-bottomed. Next, 75 µL of 0.1M sodium phosphate pH 5.6 with ANS (8.88 µM) were added on top. Peptide self-assembly was allowed to occur at room temperature for 1 hour. Then, fluorescence measurements were carried out with λ_{ex} = 360 nm, and readings in the 400-600 nm range. Samples without peptides which served as reference were prepared under the same conditions.

S13. Molecular models

Molecular models of parallel beta-sheet like structures of hFF and of a 1:1 mixture of hFF and Hff were realized by manually placing 6 peptides adjacent to each other (alternated in the case of the racemic mixture) so as to optimize intermolecular backbone H-bonds.

The models were then subjected to a three-step short energy minimization in order to optimize interactions and remove steric clashes. First, up to 5000 cycles of optimization were performed while applying harmonic restraints (k = 2.5 kcal mol^{-1} Å^{-1}) to all non-hydrogenous atoms of the backbone. Next, up to 5000 cycles of optimization were performed by applying the same restraints to the sidechain atoms. Finally, up to 1000 cycles of structural optimization were allowed in the absence of any restraint.

The parameters for the amidated C-terminal phenylalanine were built following guidelines from the AMBER website (http://ambermd.org/tutorials/basic/tutorial5/, accessed January, 2025) using the ff19SB AMBER force field[1] as for the standard peptides.

(1) Tian, C.; Kasavajhala, K.; Belfon, K. A. A.; Raguette, L.; Huang, H.; Migues, A. N.; Bickel, J.; Wang, Y.; Pincay, J.; Wu, Q.; Simmerling, C. ff19SB: Amino-Acid-Specific Protein Backbone Parameters Trained against Quantum Mechanics Energy Surfaces in Solution. *J. Chem. Theory Comput.* **2020**, *16* (1), 528–552. https://doi.org/10.1021/acs.jctc.9b00591.